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Characterization of T follicular helper (Tfh) cells and B cell isotype switching induced by type 1 and type 2 adjuvants

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DISSERTAÇÃO PARA A OBTENÇÃO DE GRAU DE MESTRE EM CIÊNCIAS BIOMÉDICAS, ESPECIALIDADE EM BIOLOGIA MOLECULAR EM SAÚDE TROPICAL E INTERNACIONAL

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Resumo

A principal função dos linfócitos T $CD4^+$ é fornecer apoio a outras células no sentido de gerar uma resposta imunitária eficiente. As interações entre as células T e B são essenciais para a produção de respostas humorais, sendo que foi recentemente demonstrado que as células T foliculares auxiliares (Tfh) desempenham um papel crucial neste processo. Caracteristicamente, expressam o fator de transcrição Bcl-6, o recetor de quimiocinas CXCR5 e o marcador de superfície PD-1. A expressão destes marcadores é única e fundamental para que estas células possam aceder ao folículo de células B, onde orientam as reações no centro germinativo (GC), levando à consequente mudança de isótipo, maturação da afinidade, produção de anticorpos de alta afinidade e células B de memória.

Neste projeto, foram testadas duas hipóteses opostas no sentido de caracterizar fenotipicamente as células Tfh. Propomos investigar se estas são especializadas no fornecimento de auxílio do tipo Th1 ou Th2, que designamos de células hipotéticas "Tfh1" e "Tfh2" (Hipótese 1) ou se são uma subpopulação genérica que responde igualmente na presença de diferentes antigénios, células Tfh (Hipótese 2).

Deste modo, murganhos C57BL/6J e Balb/c foram imunizados na almofada plantar da pata traseira, utilizando proteína Ovalbumin (OVA) combinada com diferentes tipos de adjuvantes: CpG ODNs isoladamente e em combinação com TiterMax® Gold (TMX), Sigma Adjuvant System (SAS) e Montanide ISA 720 VG, testados como adjuvantes tipo 1, e por sua vez Incomplete Freund's Adjuvant (IFA) e Alum experimentados como adjuvantes do tipo 2. A técnica de ELISA permitiu determinar no soro dos murganhos o tipo de resposta gerada, através da medição de imunoglobulinas específicas para OVA (IgG2a para Th1, IgG1 e IgE total para Th2). CpG ODNs e IFA foram considerados como os adjuvantes mais apropriados para induzir respostas Th1 e Th2, respetivamente.

Células T que reconhecem especificamente OVA foram colhidas de murganhos OT-II $Rag^{-/-}$ e DO11.10 $Rag^{-/-}$ e transferidas para murganhos congénicos. De seguida, procedeu-se à imunização tal como descrito acima. Os nódulos linfáticos drenantes foram recolhidos no pico da reação do centro germinativo (11 dias após imunização), assim como as células Tfh específicas para OVA ($CD4^+CD44^+$ $CXCR5^+PD\text{-}1^+$ $Thy1.2^+V_\beta5^+V_\alpha2^+/DO11.10^+$) e as células T auxiliares ativadas específicas para OVA ($CD4^+CD44^+CXCR5^-PD\text{-}1^-Thy1.2^+V_\beta5^+V_\alpha2^+/DO11.10^+$).

A caracterização molecular destas populações de células T está a ser analisada através da sequenciação dos seus transcritos pela técnica de *RNA-sequencing*. Além disso, a expressão de marcadores de Th1 e Th2 em células Tfh foi analisada através de citometria de fluxo e Reação em Cadeia da Polimerase quantitativa por Transcrição Reversa (RT-qPCR). Neste estudo, foi demonstrado que as células Tfh co-expressam Bcl-6 e T-bet e também produzem IFN-γ, quando sensibilizadas com OVA-CpG ODNs, características concordantes com os marcadores fenotípicos de uma célula Tfh e célula Th1. A expressão de Gata-3 (marcador Th2) só foi detetada sob estimulação IFA-OVA, embora em níveis mais baixos do que as determinadas para T-bet.

Palavras-chave: Células Th1 e Th2, Adjuvantes, células Tfh, hipotéticas "Tfh1" e "Tfh2", *RNA-Sequencing*.

Abstract

The major function of $CD4^+$ T cells is to provide help to other lymphocytes to mount an efficient immune response. T and B cell interactions are essential for humoral responses and it was recently shown that T follicular helper (Tfh) cells play a crucial role in this process. They characteristically express the transcription factor Bcl-6, chemokine receptor CXCR5 and PD-1. These markers are unique as their expression is pivotal to acquire access to the B cell follicle and drive germinal centre (GC) reactions, leading to isotype switching, affinity maturation, and production of high affinity antibodies and memory B cells.

In this project, two competing hypothesis investigating the phenotype of Tfh cells were tested. We propose to dissect whether Tfh cells are specialized in providing Th1 or Th2 help, which we call putative "Tfh1" and "Tfh2" cells (hypothesis 1), or if they are a more generic Th subset that responds equally in the presence of different antigens, which we designate as Tfh cells (hypothesis 2).

Therefore, we immunized C57BL/6J and Balb/c mice in the footpad using Ovalbumin (OVA) protein combined with different adjuvant types: CpG ODNs only and combined with TiterMax® Gold (TMX), Sigma Adjuvant System (SAS) and Montanide ISA 720 VG, as type 1 adjuvant, and Incomplete Freund's Adjuvant (IFA) and Alum as type 2 adjuvants. Using ELISA assays to determine the type of response generated by measuring serum immunoglobulins of distinct clones (OVA-specific IgG2a for Th1 and OVA-specific IgG1 and total IgE for Th2), we considered CpG ODNs and IFA as the most appropriate adjuvants to induce Th1 and Th2 responses, respectively.

OVA-specific cells were transferred from OT-II $Rag^{-/-}$ and DO11.10 $Rag^{-/-}$ mice into congenic mice subsequent to immunization as described above. Draining LNs were collected at the peak of the GC reaction (day 11 post-immunization) and OVA-specific Tfh cells ($CD4^+$ $CD44^+$ $CXCR5^+PD\text{-}1^+$ $Thy1.2^+V_\beta5^+V_\alpha2^+/DO11.10^+$) and OVA-specific activated-Th cells ($CD4^+$ $CD44^+$ $CXCR5^-PD\text{-}1^-$ $Thy1.2^+V_\beta5^+V_\alpha2^+/DO11.10^+$) were sorted.

The molecular signature of these T cell populations is being analysed via RNA-Sequencing. Moreover, the expression of Th1 and Th2 markers on Tfh cells was investigated via flow cytometry and Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR). In this study, it could be shown that Tfh cells of mice immunized with OVA-CpG ODNs co-expressed Bcl-6 and T-bet and also produced IFN-γ, both concordant features with the phenotypic markers of a Tfh cell and of a Th1 cell. As for the expression of Gata-3, it has only been detected in mice under IFA-OVA stimulation, even though at levels lower than the ones determined for T-bet.

Keywords: Th1 and Th2 cells, Adjuvants, Tfh cells, putative "Tfh1" and "Tfh2", RNA-Sequencing

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Abbreviations list

ACK	Ammonium-Chloride-Potassium
APC	Antigen-presenting cell
Bcl-6	B-cell lymphoma 6
BCR	B cell receptor
BFA	BrefeldinA
Blimp-1	B lymphocyte-induced maturation protein-1
BM	Bone marrow
BM-DC	Bone marrow-derived dendritic cells
CCR	CC chemokine receptor
CD40L	CD40 ligand
cDNA	complementary Deoxyribonucleic Acid
cTfh	circulating T follicular helper cell
CXCR	CXC chemokine receptor
DC	Dendritic cell
DZ	Dark zone
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
FDC	Follicular dendritic cell
GC	Germinal Centre
GM-CSF	Granulocyte Macrophage Colony-stimulating factor
HRP	Horseradish Peroxidase
ICOS	Inducible co-stimulator
IFA	Incomplete Freund’s adjuvant
IFN	Interferon
Ig	Immunoglobulin
IGC	Instituto Gulbenkian Ciência
IL	Interleukin
IMM	Instituto de Medicina Molecular
i.v	Intravenous
LN	Lymph node

LPS	Lipopolysaccharides
LZ	Light zone
MALT	Mucosal associated lymphoid tissues
MHC	Major Histocompatibility complex
MPLA	Monophosphoril lipid A
NK	Natural killer cell
NKT	Natural killer T cell
OVA	Ovalbumin
PBS	Phosphate buffered saline
PD-1	Programed dead-1
PI3K	Phosphoinositide-3-kinase
PMA	Phorbol-12-myristate-13-acetate
PRRs	Pattern recognition receptors
RAG	Recombination activating gene
RNA	Ribonucleic acid
RNA-seq	Ribonucleic acid sequencing
RT	Room temperature
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SAP	SLAM-associated protein
SAS	Sigma Adjuvant System
s.c	Subcutaneous
SHM	Somatic hypermutation
SPF	Specific pathogen free
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
Tfh	T follicular helper cell
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TMX	TiterMax® Gold
TNF	Tumour necrosis factor
Tregs	Regulatory T cell

1. Introduction

1. Introduction

The immune system is a remarkably versatile defence system that specifically recognizes and eliminates a variety of antigens (1) and is comprised of two different components, the innate and adaptive immune system.

Innate immunity provides the first line of defence against infection and includes physical and chemical defensive barriers as well as cellular barriers, namely phagocytes (macrophages and dendritic cells), granulocytes (neutrophils, eosinophils, basophils or mast cells) and natural killer (NK) cells (1). These cells express on their surface pattern recognition receptors (PRRs), that recognize conserved pathogen-associated molecular patterns (PAMPs) (2). Phagocytic cells internalize antigens through the endocytic pathway and process them into peptides in association with major histocompatibility complex (MHC) molecules. MHC-class II are expressed mostly by antigen-presenting cells (APC), manily macrophages, dendritic cells (DCs), and B cells. These cells are professional in antigen processing and presentation, both critical for T cell activation (1,3). Furthermore, innate activated cells produce a myriad of inflammatory cytokines and chemokines pivotal for the polarization of the appropriate subsequent immune response, directly impacting on effector function of T cells (1,2).

Adaptive immunity relies on lymphocytes to ensure specific long-lasting immunity, thereby protecting the host from subsequent exposure to the same antigen. An effective adaptive immune response is orchestrated by two major lymphocyte populations—B cells and T cells (1). Naïve B cells develop in the bone marrow (BM) and exit to circulate in blood and lymph, towards secondary lymphoid organs, where immune reactions take place. B cells can recognize soluble antigens through the B-cell receptor (BCR) with a single antigenic specificity (1,3). T cells develop and mature in the thymus and exit to the periphery as $CD4^+$ T helper (Th) or $CD8^+$ cytotoxic T (Tc) cells, the two main effector T cell populations of the αβ lineage. Unlike B cells, the T-cell receptor (TCR) can only recognize antigens that are presented by MHC molecules, with $CD4^+$ T cells and $CD8^+$ T cells recognizing antigen bound to class II MHC molecules or to class I MHC molecules, respectively (1–3).

1. Lymphoid organs

Lymphoid organs can be largely divided into primary and secondary lymphoid organs (1–3). Primary lymphoid organs, the thymus for T cells and the BM for B cells, are the place of lymphoid development. On the other hand, the morphologically and functionally distinct secondary lymphoid organs, constitute the sites where specific adaptive immune responses take place, through the presentation of a given antigen by APCs, resulting in the recruitment, activation and maturation of responding effector T cells and B cells.

1.1 Secondary lymphoid organs

The secondary (or peripheral) lymphoid organs are the spleen, lymph nodes and various mucosal associated lymphoid tissues (MALT), which trap antigen and provide places for interaction between mature naïve lymphocytes and its cognate antigen (4).

Lymph nodes are extremely organized structures, interconnected by a system of lymphatic vessels, which drain lymph, an extracellular fluid continuously produced through filtration of the blood and tissues. Morphologically, they can be divided into three roughly concentric regions, each supporting a distinct microenvironment: the cortex, the paracortex and the medulla (1). The cortex is arranged in primary follicles that contain mostly B cells, macrophages, and follicular dendritic cells (FDCs). The primary follicles enlarge, upon antigenic challenge, into secondary follicles, each containing a germinal centre. The paracortex, located between the cortex and the medulla, is populated largely by T cells and also interdigitating dendritic cells. These cells express high levels of MHC class II which are necessary for presenting the antigen, trapped in tissues and then drained, to T helper cells. The medulla is the innermost layer of a lymph node, formed mainly by plasma cells actively secreting antibody (1–4).

1.2 Germinal centre

Germinal centres (GC) are dynamic microenvironments of secondary lymphoid organs that provide a unique niche for B-cell affinity maturation (5). GCs develop only in response to an antigen and are initially engaged by migration of antigen-activated B cells that expand at around 6 days after a primary immunization (6). In GCs two distinct areas were identified, the dark zone (DZ) containing B cells and the light (LZ) zone that contains a rich network of FDCs and T follicular helper cells (Tfh) (7) (Figure 1.1).

Affinity maturation takes place in B cells that actively proliferate in the DZ of GCs. These cells express high levels of the chemokine receptor CXCR4 that allows their maintenance in the DZ, in response to chemokine CXCL12 produced by stromal cells (8). The DZ B cells, known as centroblasts, then reduce expression of surface immunoglobulin (Ig) and undergo somatic hypermutation (SHM) of heavy- and light-chain variable region genes (5,9). Somatic hypermutation is induced by the enzyme cytidine deaminase (AID) and occurs randomly. Consequently, cells expressing receptors (BCR) of unchanged or lower affinity will be generated, as well as cells with receptors of higher affinity for a particular antigen (5) (Figure 1.1).

These cells also suffer class switch recombination, a biological mechanism that allows the change of the constant region of the antibody-heavy chain and, consequently, the alteration of the antibody class produced by an activated B cell (10). Naïve B cells express the IgM isotype and begin to express both IgM and IgD when they reach maturity. If antigen-activated B cells encounter specific signalling molecules via their CD40 and cytokine receptors, they undergo antibody class switching to produce IgG, IgA or IgE antibodies, allowing interaction with different effector molecules that have defined roles in the immune system (10).

Then, surviving DZ B cells will then upregulate the chemokine receptor CXCR5 and migrate toward the LZ. There, FDCs produce CXCL13, the ligand for CXCR5 and the major chemoattractant in the LZ (11). At this stage B cells, called centrocytes, stop dividing, increase the expression of surface Ig and are selected by the ability to recognize and bind antigen displayed on the surface of FDCs (6). Furthermore, the centrocyte must also receive survival signals from interaction with cognate Tfh cells (9) (Figure 1.1).

B cells that successfully interact with FDC and Tfh cells can follow three potential fates. First, GC B cells can upregulate CXCR4 to undergo recycling and re-entry into the DZ, for further proliferation and SHM. Second, GC B cells can also differentiate into memory B cells that stop dividing and enter the G0 phase of the cell cycle, during a primary response. Finally, cells can exit the GC as a plasma cell. As a consequence of SHM and class switching into the DZ, plasma cells produce higher antigen-affinity and express additional antibody isotypes, including IgG, IgA, and IgE (12). Additionally, differentiation of antibody-secreting plasma cells involves changes in gene expression that allows the gain of protein production and secretion functions. Such modifications are mediated by the master transcription factor B lymphocyte-induced maturation protein-1 (Blimp-1) (13).

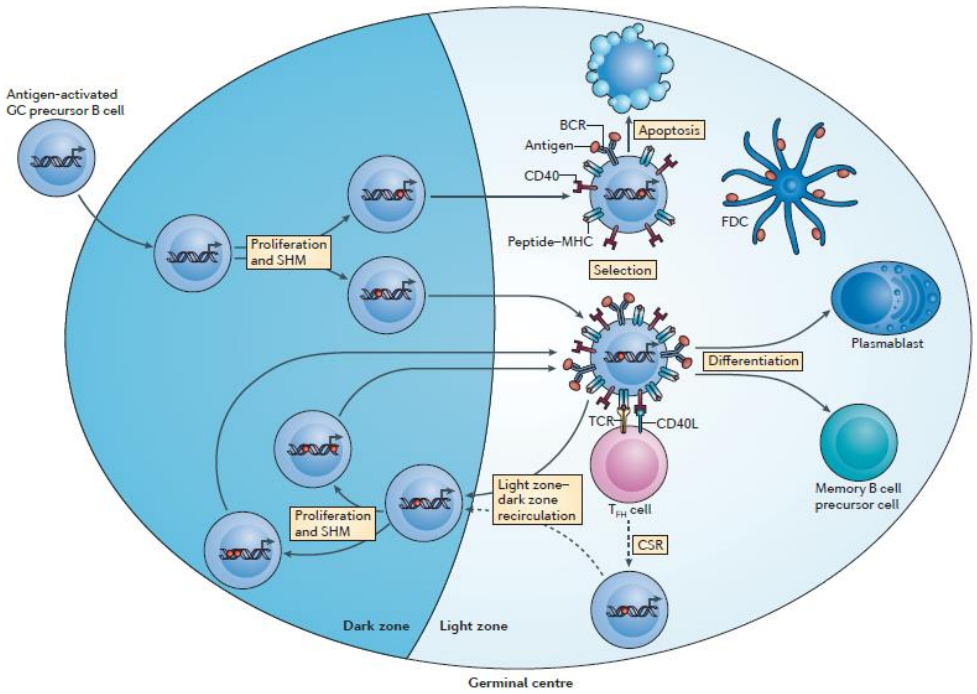


Figure 1.1- Compartments and dynamic of the GC. Centroblasts undergo somatic hypermutation and clonal expansion into the DZ. During proliferation, the process of SHM introduces changes into the V(D)J region of the rearranged immunoglobulin variable region (IgV) genes. Dark zone B cells then move to the LZ, where the modified BCR is selected for improved binding to the immunizing antigen. Tfh and FDCs participates actively in this process (7).

GC B cells can be identified by their expression of high levels of death receptor Fas and *n*-glycolylneuraminic acid (the ligand of antibody GL-7), loss of surface IgD and anti-apoptotic molecule Bcl-2 (6). The transcriptional factor Bcl-6 is selectively upregulated during the GC stage as the critical regulator in the generation of the GC B cell phenotype.

2. T helper cells ($CD4^+$ cells)

$CD4^+$ T helper (Th) cells play a critical role in providing help to other lymphocytes and innate immune cells to mount an efficient immune response. $CD4^+$ T cells have the capacity to secrete appropriate cytokines and express a variety of essential co-stimulatory molecules, important for their effector functions (14). Th cells carry out multiple tasks, ranging from activation and recruitment of other cells, including neutrophils and monocytes, B-lymphocytes, cytotoxic T cells, as well as non-immune cells. They also play critical role in the suppression of immune reaction (14).

2.1 CD4 T cell differentiation

The initial step of naïve $CD4^+$ T cells differentiation is the antigenic stimulation through interaction of TCR with antigen-MHC II complex, presented by APCs. It consequently induces a network of signalling pathways which lead to cell proliferation and differentiation into specific effector cells. Lineage-specific differentiation depends on the cytokine milieu of the microenvironment as well as on the concentration of antigens, type of APCs, and co-stimulatory molecules (15). Co-stimulatory signals enhance TCR signal, and the main co-stimulatory receptor is CD28 expressed in all naïve T cells. The CD28 ligands, CD80 and CD86, are upregulated upon activation of DCs. The initial source of cytokines are from the APCs as well as other members of the innate immune system. Subsequently, $CD4^+$ T cell differentiation into various effector subtypes (16) is coordinated by genetic programs and epigenetic modifications, triggered by appropriate transcription factors to direct expression of distinct soluble mediators and surface molecules that support interactions with other immune cells. Besides the classical Th1 and Th2, other subsets have been identified, including Th17, regulatory T cell (Treg) and Tfh cells, each with a characteristic profile and function (17).

Th1 cells

Interleukin 12 (IL-12) and interferon γ (IFN-γ) are both critical cytokines for initiating the signalling cascade to develop Th1 cells (16). IL-12 is secreted largely by APCs, after their activation through the PRRs (15), and, in turn, IL-12 induces NK cells to produce IFN-γ.

T-bet is the principal master regulator that, in coordination with other transcription factors, induces full differentiation of Th1 cells. T-bet enhances the production of IFN-γ which actively stimulates the signal transducer and activator of transcription 1 (STAT-1). T-bet expression was found to be strongly dependent on STAT-1. T-bet further induces IFN-γ and TNF-α production by the differentiating cells, thereby amplifying T-bet expression and upregulating the expression of IL-12 receptors (IL-12R). Then, cells can be selected by the abundant IL-12 from the APCs, ensuring selective expansion of the differentiating Th1 cells (15).

IFN-γ stimulates the microbicidal activity of macrophages, up-regulates the level of class II MHC and increases the production of more IL-12 (18). Furthermore, IFN-γ produced by Th1 cells also induces antibody-class switching to some IgG classes, with production of IgG2a (in mice). The IgG2a isotype has been shown to be particularly potent in host defence against viral infections due to its ability in binding to Fc receptors expressed on phagocytes, activating the complement system and inducing Ab-dependent cell mediated cytotoxicity (19).

Importantly, T-bet suppresses the development of Th2 cells by inhibiting the crucial IL-4 gene and impairing the function of the Th2 master regulator Gata- 3 (20). Th17 lineage is also inhibited in consequence of the interaction between T-bet and Rorc promoter, which encodes RORγt, the principal transcription factor of Th17 cells.

The Th1 subset is responsible for many cell-mediated functions (e.g. promoting differentiation of $CD8^+$ cytotoxic T cells, that perform an essential role in inhibiting replication of intracellular pathogens such as viruses) and for the production of opsonizing IgG antibodies (i.e. antibodies that bind to the high-affinity Fc receptors of phagocytes and interact with the complement system). This subset is also associated with the promotion of excessive inflammation and tissue injury (1,2,18).

Th2 cells

IL-4 and IL-2 are the critical cytokines for Th2 differentiation. IL-4 upregulates the expression of the major transcription factor involved in Th2 lineage, the master regulator Gata-3, which consequently enhances the production of IL-4, IL-5, and IL- 13 (1,2).

STAT-5 is readily activated by IL-2 and has an important role in Th2 lineage commitment. For full differentiation of Th2 cells, the coordinated activity of STAT-5 and Gata-3 is required, since they bind to different sites of the IL-4 locus.

IL-4 promotes a pattern of class switching that produces neutralizing IgG1 (in mice) and IgE by activating transcription factors such as STAT-6, and also inhibits IgG2a and IgG2c isotypes in mice (16). IL-13 can also contribute to IgE class switch that plays a role in eosinophil-mediated defence, because eosinophils express Fc receptors specific for IgE and some IgG antibodies. IgE is essential against helminth infections and is also the principal mediator of allergic reactions (21).

Moreover, IL-4 and IL-13 induce alternative macrophage activation and induce secretion of growth factors that stimulate fibroblast proliferation and collagen synthesis, which contributes to tissue repair and fibrosis. IL-13 stimulates mucus production by airway epithelial cells, an important component of allergic reactions. IL-5 is important for eosinophil activation, growth and differentiation, and serves as the principal association between T cell activation and eosinophilic inflammation (1,2).

Gata-3 also inhibits Th1 differentiation, presumably through interaction with T-bet, downregulating the expression of STAT-4 and the signalling chain of the IL-12R (15). Th2 cells are involved in allergic diseases, promoting airway inflammation, eosinophilia, asthma and exacerbation of helminth infections (22,23).

Th17 cells

IL-6, IL-21, IL-23, and TGF-β are the major signalling cytokines involved in Th17 cells differentiation, and retinoic acid receptor-related orphan receptor gamma-T (RORγt) is the master regulator (15). IL-6, IL-21 and IL-23 activate STAT-3 signalling that plays an important role in RORγt expression and Th17 differentiation process.

These cells appear to be abundant in mucosal tissues, particularly of the gastrointestinal tract, suggesting that Th17 cells may be specialists in combating intestinal infections and in the development of intestinal inflammation. Most of inflammatory actions of these cells are mediated by IL-17, which stimulates the production of chemokines and other cytokines that recruit neutrophils to the sites of infection. IL-17 also stimulates the production of antimicrobial substances (1,2). The main effector function of Th17 cells is to control bacterial and fungal infections (24) through neutrophilic inflammation. Th17 cells have been associated to the pathogenesis of inflammatory diseases, e.g. psoriasis, rheumatoid arthritis and multiple sclerosis (25,24).

Treg cells

Regulatory T cells (Tregs) express Foxp3 and secrete anti-inflammatory cytokines, such as TGF-β and IL-10. Tregs manifest their function through numerous mechanisms that include the secretion of immunosuppressive soluble factors such as IL-9, IL-10 and TGF-β, cell contact mediated regulation via the high affinity TCR and other costimulatory molecules (26,27).

Naturally occurring $Foxp3^{+}CD4^{+}CD25^{+}$ Treg cells develop in the thymus and display a diverse TCR repertoire that is specific for self-antigens (28) However, Treg cells can also be induced from effector T cells during inflammatory processes in peripheral tissues (29).

Regulatory T cells (Tregs) are critical to maintain immune homeostasis, limiting the magnitude of immune response against pathogens and control inflammatory and autoimmune reactions (26,27,30).

Tfh cells

T follicular helper cells (Tfh) express Bcl-6 as the major transcription factor and produce IL-21. These cells are specialized in providing support to B cells during the GC reaction, essential for the generation of high-affinity memory B cells and antibody-producing plasma cells (31). Tfh cells will be discussed in detail in the following section.

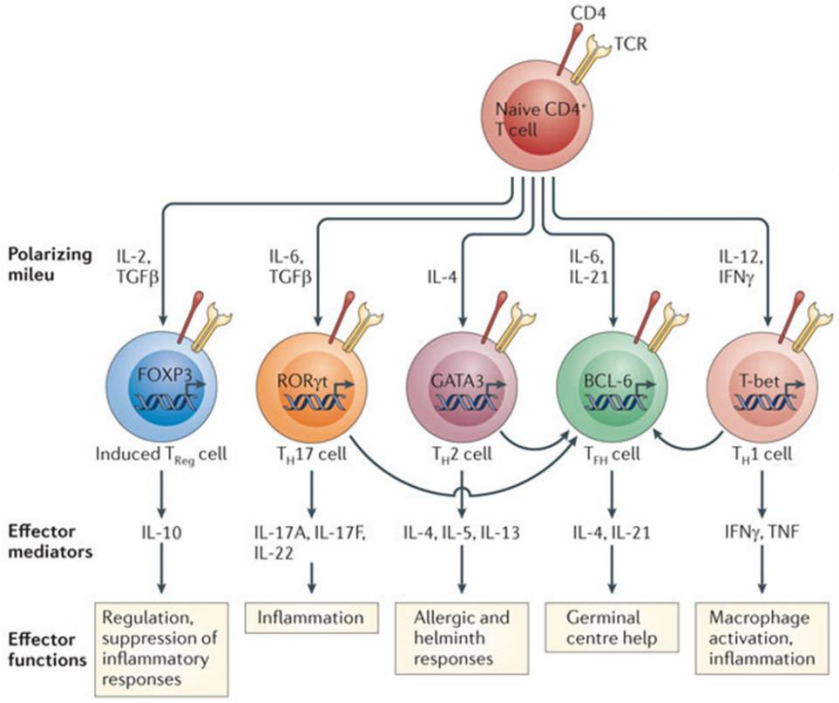


Figure 1.2- Major pathways of naïve CD4+ T cell differentiation into effector cells. Upon encountering antigens presented by the professional APCs, naïve $CD4^+$ T cells differentiate into Th1, Th2, or Th17 effector cells. Cytokines present in the environment during differentiation play the major role in determining the phenotype that the $CD4^+$ T cell will acquire (32).

3. T follicular helper (Tfh) cells

In the early 2000s, a new population of $CD4^+$ T cells was reported in human tonsils and also in lymphoid organs of immunized mice (33). The original defining feature of this designated Tfh cell was the expression of the chemokine receptor CXCR5, which was found to be indispensable for the co-localization of cognate T and B cells, which can interact during GC reactions (34). However, the original description of Tfh as $CXCR5^+$ cells was insufficient to establish it as a distinct lineage of CD4 T cells. In 2009, B-cell lymphoma-6 (Bcl-6) was described by three independent groups as the master transcription regulator of Tfh cells (35–37). Tfh cells are pivotal for germinal centre formation, providing help and support to B cells while undergoing affinity maturation and class switch recombination, culminating in the secretion of high affinity antibodies, plasma cells and memory B cells.

3.1 Phenotypic features of Tfh cells

Tfh cells express a range of cell surface molecules that are essential for their identification and allow their functions in their interactions with B cells. As referred above, Tfh cells express high levels of CXCR5 that, together with down-regulation of CCR7, facilitates their migration into B cells follicles, in response to CXCL13 (11). This co-localization is critical for T-B interactions and important for proper generation of functional Tfh cells (38).

Typically, Tfh cells are also identified by the co-expression of other surface markers, most commonly programmed death-1 (PD-1) and inducible co-stimulator (ICOS). Both these molecules are members of the CD28 family and are up-regulated on T cells following activation. PD-1 is a negative regulator that provides an inhibitory signal to GC B cells, that express PD-1 ligand 1 or 2, preventing excess of Tfh cells proliferation in GC (31). PD-1 and PD-1 ligand deficient mice displayed higher frequencies of Tfh cells after protein immunization (39), alongside worsened B cell responses due to increased GC B cell apoptosis (39).

ICOS is a co-stimulatory molecule expressed on activated T cells, whereas its ligand ICOSL exists on B cells, macrophages and other APCs. Several studies have demonstrated that ICOS signalling seems to be dependent on its ability to activate phosphoinositide-3-kinase (PI3K) signalling (40). ICOS is able to up-regulate Tfh cell-associated genes such as c-Maf, IL-4 and IL-21. This plays an important role in Tfh cell generation, GC formation and antibody production (41).

Additional surface molecules are importantly expressed by Tfh cells. CD40 ligand (CD40L) provides survival signals to B cells through CD40 molecule and induces B cell differentiation and class-switching. The signalling adaptor SLAM-associated protein (SAP) plays an indispensable role for stable T and B interactions required for Tfh cell differentiation.

IL-21 is produced by activated $CD4^+$ T cells and NKT cells and are highly expressed by Tfh and GC Tfh cells (42). This cytokine plays a major role in Tfh cell survival and GC B cell proliferation, survival and differentiation (42). IL-21 is the most potent inducer of plasma cell differentiation in both mice and humans, a process that is STAT-3 dependent.

Additionally, IL-21 can induce expression of Blimp-1 and Bcl-6 transcription factors on B cells in a mutually exclusive manner (43).

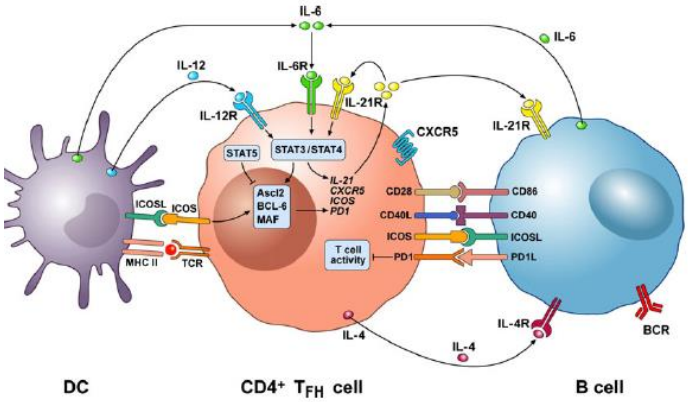


Figure 1.3- Cellular and molecular interactions between DCs, Tfh and B cells in GC. Tfh cells express a range of surface molecules, ICOS, CD28, CD40L and PD-1, important for interaction with DC in the T-cell zones and B cells in GC of lymph nodes (44).

3.2 Bcl-6 as Master Regulator of Tfh cells differentiation

Like other Th lineages, Tfh cells are associated with expression of a canonical transcription factor. Bcl-6 was identified as a master regulator transcription factor in Tfh cell differentiation. T cells deficient in Bcl-6 are unable to differentiate into Tfh cells or to sustain GC reactions, while Bcl-6 overexpression facilitates the expressions of Tfh-associated molecules such as CXCR5 and PD-1 (45).

Bcl-6 appears to participate in control of at least four major categories of genes: genes involved in cell migration, repression of alternative fates, Tfh differentiation and Tfh cytokines production (37). Bcl-6 controls GC B cell differentiation by regulating cell cycle genes and DNA damage response genes, supressing a host of signalling pathways, including BCR signalling (36,46,47). Tfh cells express high levels of Bcl-6 while non-Tfh cells (i.e Th1, Th2, Th17) express Blimp-1. Blimp-1 is a reciprocal antagonist of Bcl-6 and can inhibit Tfh cell development (35). Importantly, Bcl-6 can antagonize transcription factors important for Th1 (T-bet), Th2 (Gata-3) and Th17 (RORγt) differentiation. However, this inhibition can be, and is most frequently, incomplete.

3.3 Tfh cell differentiation

In 2011 Shane Crotty proposed a canonical Tfh cell differentiation model, i.e. a multistage and multifactorial process (31) involving several events, molecules and two different cells as APCs. Nowadays, although much more is understood about the multiple stages and signals involved in the process, critical knowledge is still lacking because it has not been possible to reproducibly mimic Tfh cell differentiation *in vitro*. Tfh cell differentiation comprises four different stages, with characteristic molecules, signals and APCs playing essential roles for a successful process culminating in the development of a fully differentiated Tfh cell.

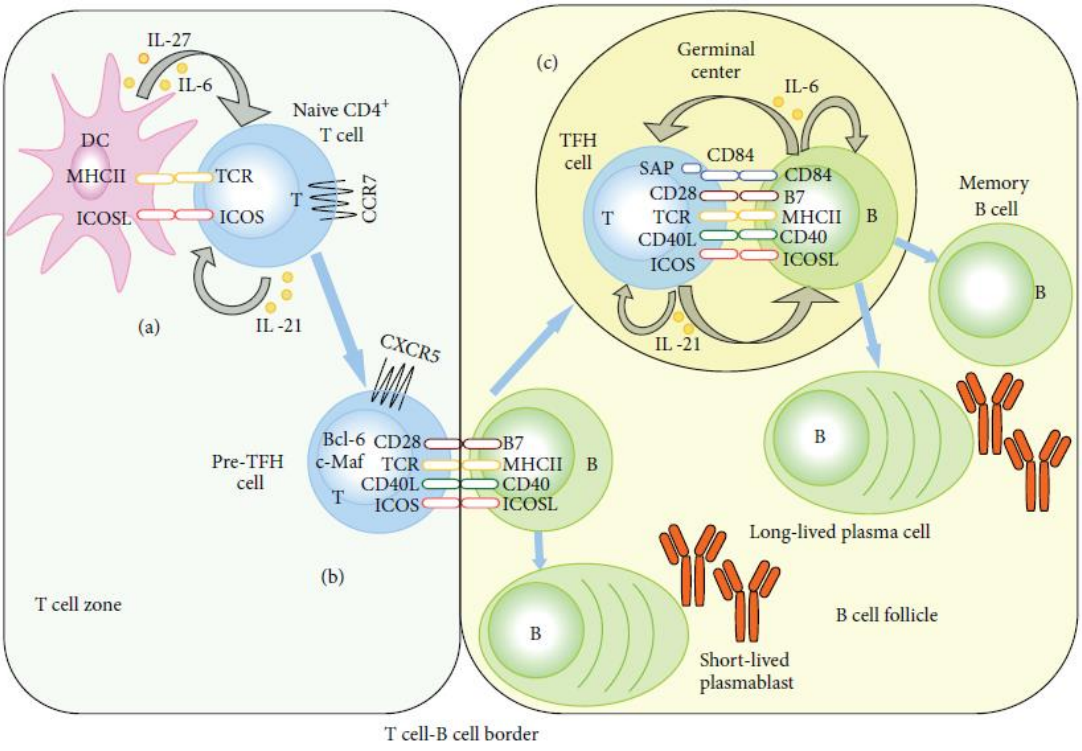


Figure 1.4- Canonical model of Tfh cell differentiation. Multiple signals and steps for the generation of Tfh cells. **(a)** Within the T cell zone, naïve $CD4^+$ T cells encounter DCs, activating T cells that move towards B cell follicles. **(b)** At the T-B border, activated T cells become pre-Tfh cells interacting with cognate activated B cells. **(c)** In the germinal centre, pre-Tfh cells become Tfh cells and provide help for B cell differentiation into plasma cells and memory B cells. Tfh cell differentiation involves a series of co-stimulatory molecules and cytokines, which are important for their function in providing help to B cells (45).

3.3.1 Stage 1: Generation of pre-Tfh

After an infection or immunization, naïve $CD4^+$ T cells in the T cell zone interact with antigen presenting-DCs. Naïve $CD4^+$ T cells acquire features of pre-Tfh or early Tfh cell and undergo a cell fate decision within the first few rounds of cell division (38). The increased expression of Bcl-6 and downregulation of antagonist Blimp-1 is taking place during this early stage, within 3 days after immunization or infection. This program is influenced by IL-6 and IL-21 (in mice) or IL-12 (in humans) and co-stimulation through ICOS and CD28 (36). ICOS plays a role in both Tfh cell differentiation and migration, and there is data supporting a synergistic role of ICOS and IL-6 (40) (Figure 1.4). The transcription factor Bcl-6 is crucial for maintenance of the Tfh program, including the expression of CXCR5 and the downregulation of CCR7. Thus, the early Tfh cell will migrate to the border of the B cell follicle and undergo further Tfh cell differentiation (48). Thus, the interplay between IL-6, ICOS, IL-21, and TCR signaling orchestrates early induction of mouse Tfh cell differentiation during DC priming via control of CXCR5, Bcl6, and other targets (49) (Figure 1.4).

3.3.2 Stage 2: Guiding pre-Tfh into GC

The second stage of Tfh cell differentiation occurs in the T-B border, where pre-Tfh cells contact with cognate activated B cells, which serve both as APCs and as a source of ICOSL (48,49). The stable T-B conjugates so formed move into the GC, while low affinity T cells that fail to interact with their cognate B cell, do not gain access to the GC and, expectedly, accumulate in the T-B border (31,45) (Figure 1.4).

3.3.3 Stage 3: Tfh differentiation in GCs

The third stage occurs in the GC, where pre-Tfh cells finally differentiate into Tfh cells which help GC B cells during affinity maturation, class switch recombination and differentiation into memory B cells or plasma cells (50). Tfh cells express high levels of CXCR5, PD-1, Bcl6 and ICOS (31,37) (Figure 1.4). Moreover, SAP expression is also essential for Tfh cell development, GC development, and the generation of the majority of memory B cells and memory plasma cells (31,51).

In the absence of SAP, Tfh cells display defective adhesion to GC B cells and fail to be retained in GCs. In addition, IL-21 produced by Tfh cells prompts their own differentiation, while IL-6 produced by B cells is important for Tfh cells maintenance (49) (Figure 1.4).

3.3.4 Stage 4: Tfh cells can exit GCs

After Tfh cells differentiation, they can exit the GC in several ways. Tfh cells can transit to neighboring follicles and enter a different GC (52), or temporarily reside in an adjacent B cell follicle before re-entering the same GC. Alternatively, Tfh cells can downregulate Bcl-6 and develop into a memory Tfh cell (53). Tfh memory cells are characterized by intermediate expression of CXCR5 and CCR7. Despite the fact that Bcl-6 expression is not detected in memory Tfh cells, c-Maf and other transcription factors are maintained at low or intermediate levels. Furthermore, ICOS, IL-21 and PD-1 are absent in antigen-specific memory Tfh cells in mice. One study has demonstrated that antigen-specific memory cells expressing CXCR5, provided accelerated B cell responses and antibody class switching. The authors suggested that CXCR5 expression promotes their rapid migration to the GC (53).

3.4 The transcriptional signature of Tfh cells

The transcriptional profiling of Tfh cells has revealed a distinct repertoire of expressed genes that distinguish them from Th1, Th2, or Th17 cells (54). However, it is not yet defined whether Tfh cells can be themselves sub-divided into distinct populations, which can provide specific help to B cells, depending on antigen type.

Recently, few studies have reported that Tfh cells in GCs have the capacity to produce either IFN-γ or IL-4, depending on how they were primed (31,35,55). Nevertheless, this fact does not distinguish a scenario where a cell of the Tfh lineage subsequently acquires the capacity to produce IL-4 or IFN-γ, from another where Th2 or Th1 cells could acquire the capacity to act as Tfh cells (35).

This was interpreted as evidence that Tfh cells can produce cytokines associated with canonical helper T effector subsets depending on environmental conditions. According to these, further studies are lacking to clarify this question.

4. Adjuvants

Immunization with purified protein antigens typically results in the induction of a modest antibody response, with little or no T cell involvement. In order to overcome this, adjuvants, i.e. compounds with the capacity to enhance the immune response against co-inoculated antigens, have been used to boost immune responses to an antigen of interest.

They can sustain and improve the immunogenicity of antigens, effectively modulating appropriate immune responses, thereby reducing the amount of inoculated antigen and/or the number of immunizations. Some of the features involved in adjuvant selection are the particular antigen, the formulation and safety aspects, the animal species, the route of administration and, importantly, the type of immune response, or more appropriately, the phenotype of the effector T and B cells so generated. In a nutshell, adjuvants should promote an appropriate immune response, stimulating both cellular and humoral immunity.

Adjuvants can be classified according to their component sources, physiochemical properties or mechanisms of action (56). Adjuvants usually fall in two major functional groups, based on being dependent (Type 1) or not (Type 2) on Toll-like-receptors (TLRs) signalling (Figure 1.5).

Type 1 adjuvants are recognised by TLR and directly act on the immune system to increase responses to an antigen, while type 2 adjuvants, present antigens in an optimal manner, including controlled release and depot delivery systems, thereby enhancing the specific immune response to the immunizing antigen. (56) (Figure 1.5).

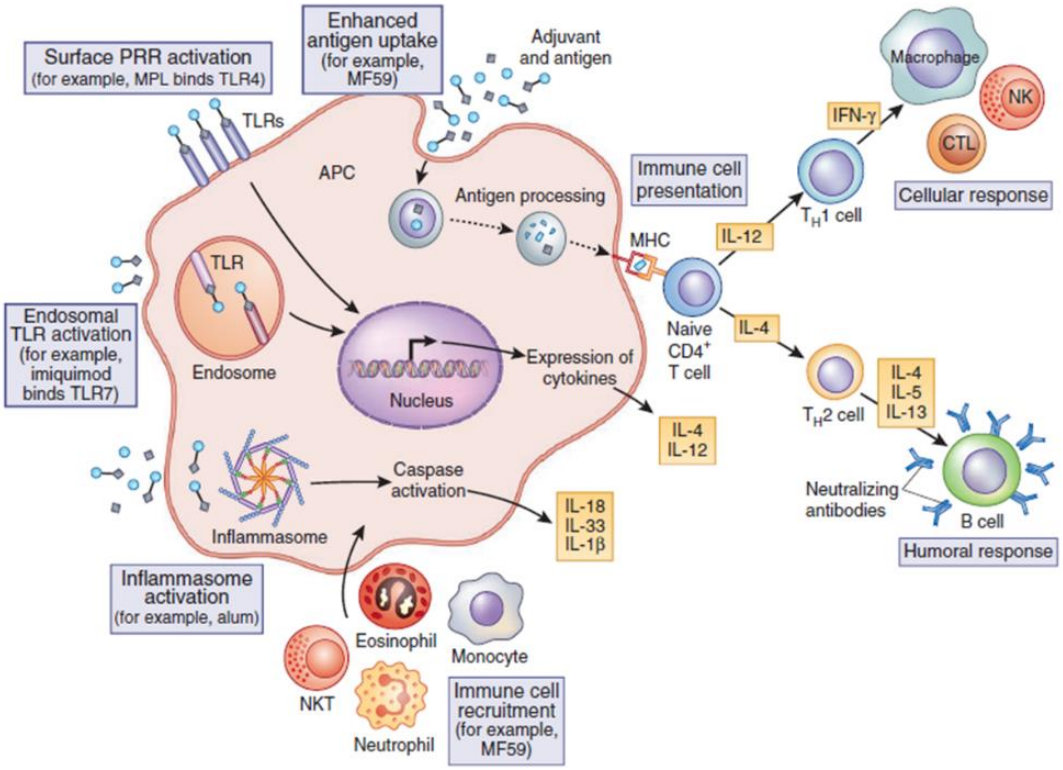


Figure 1.5- Putative mechanisms of action of adjuvants. Many adjuvants can act as ligands for PRRs that activate an innate immune response. Receptor signalling can then activate transcription factors that induce the production of cytokines and chemokines that help direct a particular immune response, such as a Th1 or Th2 type response. Some adjuvants also influence presentation of antigen by MHC (57).

2. Objectives

Tfh cell differentiation remains controversial because it is still unknown whether Tfh cells can be subdivided in different subsets what we call putative "Tfh1" or "Tfh2" (Hypothesis 1), or if they are a more "general" subset of T helper cells, Tfh (Hypothesis 2) such as Th1, Th2 or Th17. Our proposal addressed these two competing hypotheses as shown on figure 2.1.

The first hypothesis (a) postulates the existence of Tfh subsets specialized in providing Th1 or Th2-type "help", a subset we named here putative "Tfh1" or "Tfh2". The acquisition of such specialized Tfh function may be a consequence of early interaction with DCs or following interaction with B cells in the T-B border, and it may be influenced by cytokines produced by Th1, Th2 or Th17 cells.

The alternative model (b) defends that "help" provided by Tfh cells in the course of a Th1 or Th2-associated immune response is the responsibility of a non-specialized and generic cell population, Tfh cells. Thus, isotype-switch is influenced by contact with generic Tfh cells and cytokines produced in the vicinity by extra-follicular Th1 or Th2 cells.

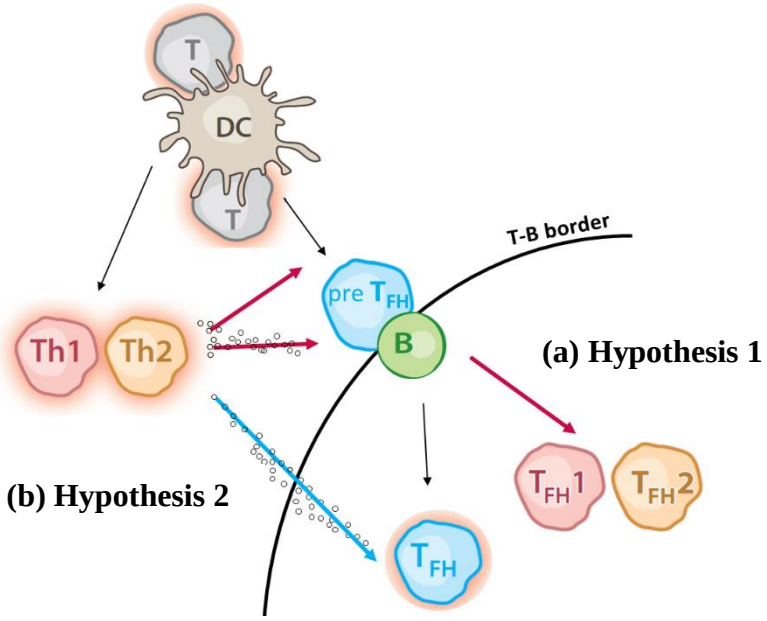


Figure 2.1- Two alternative models for Tfh cells contribution in GC reaction. **(a)** Extra-follicular environment can lead to acquisition of type-1 or type-2 characteristics by Tfh cells, originating specialized "Tfh1" or "Tfh2" cells that then drive GC formation. **(b)** As an alternative, Tfh cell contribute as non-specialized Tfh cells together with cytokines produced by extra-follicular Th1 or Th2 cells.

In this work, we aimed to characterize the phenotype of putative specialized "Tfh1" and "Tfh2" subsets, or exclude their existence, in two different genetic backgrounds, Balb/c mice or C57BL/6J mice (which will be generically designed as B6 mice). We will dissect and assign, should it exist, a molecular signature to the at presumed Tfh1 and Tfh2 cells, through RNA-sequencing analysis of purified antigen-specific cells.

To achieve this goal, the work has been organized in two tasks:

i. Identification of ideal immunizing conditions to drive Th1 and Th2 humoral responses.

Primary, mice will be subcutaneously immunized in the footpad with OVA (protein) with either a type 1 or a type 2 adjuvant to generate Th1 or Th2 immunity, respectively. We will collect serum at day 14 in order to determine the type of response generated, by measuring serum OVA-specific (IgG2a for Th1, IgG1 for Th2) and total IgE (Th2) by ELISA. We will also collect draining LNs at the peak of GC reaction (Day 11) for further evaluation of the presence of effector Th and Tfh cells, by flow cytometry.

ii. Purification of antigen-specific populations of Th1, Th2 humoral responses.

Then, using established immunizing conditions, $CD4^+$ T cells will be isolated from OVA-specific TCR-transgenic OT-II or DO11.10 mice and adoptively transferred intravenously into congenic B6 or Balb/c hosts, respectively. Mice will be subsequently immunized as described with OVA in conjugation with defined adjuvants.

Draining LNs will be collected and Tfh cells ($CD4^+CXCR5^+PD\text{-}1^+$) and extra-follicular cells ($CD4^+CXCR5^-PD\text{-}1^-$) will be identified by flow cytometry. From each condition we will sort antigen-specific Tfh cells and antigen-specific extra-follicular cells (Th1 or Th2). These T cell populations are being further analyzed by RNA-Sequencing, an objective outside the scope of this thesis.

3. Materials and methods

1. Experimental mice

Balb/c, C57BL/6 (B6), B6 Thy1.1, B6 Thy1.1/Thy1.2, DO11.10 $Rag^{-/-}$, OT-II $Rag^{+/+}$ and OT-II $Rag^{-/-}$ mice were bred and maintained in specific pathogen free (SPF) conditions at the Instituto Gulbenkian de Ciência (IGC) and Instituto de Medicina Molecular (IMM). All mice were used between 6–10 weeks of age.

2. Ethics statement

Procedures were conducted in accordance with the Portuguese and European laws (Portaria 1005/92 and Directive 2010/63/EU) and following the FELASA recommendations. Procedures were conducted in accordance with guidelines from the Animal User and Institutional Ethical Committees.

3. Immunoglobulin quantification using Enzyme-Linked Immunosorbent Assay (ELISA)

Immunoglobulin concentration in the serum was determined using ELISA assay. Serum was obtained through centrifugation (1000g, 10min, RT) from whole blood collected by cardiac puncture on the day indicated. For measurement of IgG OVA-specific antibody levels, plates were coated with 50µl/well of OVA solution (20µg/ml, Sigma Grade V, Cat. No. 5503). For detection of total IgE, the plates were coated with capture purified anti-mouse IgE monoclonal antibody (diluted 1:250, eBioscience, Cat. No. 88-50460). All reagents were diluted in ELISA coat (Table 3.1), overnight at 4°C.

Serum was serially diluted and tested as presented in table 3.2. The standard curve was obtained using a mouse anti-Ovalbumin IgG1 monoclonal antibody (AbCam, Cat. No. 17293), mouse anti-Ovalbumin IgG2a monoclonal antibody (Chondrex, Cat. No. 7095) and recombinant mouse IgE (eBioscience, Cat. No. 88-50460), starting the curve at 1µg/ml for IgG1 and IgG2a and 100ng/ml for IgE. Standards and samples were incubated on ELISA buffer (50µl/well) for a minimum of 1h30min.

As detection reagents, goat-anti-mouse-IgG1-Horseradish Peroxidase (HRP) or goat-anti-mouse-IgG2a-HRP (diluted 1:2000, SouthernBiotech) and biotin-conjugated anti-mouse IgE (diluted 1:250, eBioscience) were incubated for 45min (50µl/well, RT). Additionally, to detect IgE, plates were incubated with Streptavidin-HRP (diluted 1:400, eBioscience) for 30min (50µl/well, RT). The TMB substrate was added and the reaction was stopped with H_2SO_4 (1M, 25µl/well) and the colorimetric product was evaluated through spectrophotometric absorbance at 450nm.

Table 3.1- Solutions and substrate solution used in ELISA assay.

Solution	**Composition**
ELISA coat	Sodium carbonate, 50mM, pH 9.5
ELISA wash	Phosphate buffered saline (PBS) 1X + 0.1% (v/v) Tween-20
ELISA buffer	PBS 1X + 0.1% (v/v) Tween-20 + 1% (w/v) Bovine Serum Albumin-BSA (Sigma A3912)
TMB substrate	TMB solution, equal parts of substrate reagent A and B, BD OptEIA

Table 3.2- Serum dilutions used for the detection of IgG2a, IgG1 and IgE concentrations in B6 and Balb/c mice.

Strain	**Immunoglobulin**	**Dilution**
B6	OVA-specific IgG1	1:15000
	OVA-specific IgG2a	1:1500
	Total IgE	1:100
Balb/c	OVA-specific IgG1	1:20000
	OVA-specific IgG2a	1:20000
	Total IgE	1:100

4. Adoptive cell transfers and mice immunization

OVA-specific cells were collected from spleen and LNs of OT-II $Rag^{-/-}$ or DO11.10 $Rag^{-/-}$ mice and transferred into B6 Thy1.1, B6 Thy1.1/Thy1.2 or Balb/c recipient mice, respectively (in 100µl saline/mouse, intra venous (i.v) retro orbital injection (Day -1). Mice immunized with type 1 adjuvants received 8-10x10^6 cells/mouse, while mice immunized with type 2 adjuvants received 2-5x10^6 cells/mouse.

The following day (Day 0) mice were immunized in the hind footpads with 80µg/50µl/footpad of OVA protein (Sigma-Aldrich, Cat. No. 031M7037V), conjugated with the indicated type 1 or type 2 adjuvant (Table 3.3).

Emulsions of OVA protein conjugated with TiterMax® Gold (TMX), Sigma Adjuvant System (SAS), Montanide ISA 720 VG or Incomplete Freund's Adjuvant (IFA) were prepared mixing equal volumes of the aqueous OVA solution and of the chosen adjuvant (1:1 volume) in glass syringe with Luer Lock Tips, to allow a tight fit and the formation of a perfect emulsion. After mixing, the syringes were rested in the fridge to guarantee that the emulsions formed were stable and ready to be administered to the mice, following transfer to 1 ml plastic hypodermal syringes. The Aluminium hydroxide gel (Alum) has a white gelatinous precipitate appearance and is supplied at a 2% (v/v) concentration, with an aluminium content varying from 9.0–11.0mg/ml. Adsorption of the OVA to the Alum was performed by mixing the OVA in aqueous solution to an equal volume of Alum and slowly stirring for 30min at 4ºC. Preparation of CpG ODNs was achieved by mixing the OVA and CpG ODNs aqueous solution by vortexing for a few minutes.

Table 3.3- Adjuvants tested to induce Th1 or Th2 responses. TMX, SAS, CpG ODNs and Montanide ISA 720 VG were used as type 1 adjuvants, while Alum and IFA were tested as type 2 adjuvants.

	Adjuvant	***Vendor (Cat. No.)***	***Formulation***	***CpG ODNs /footpad***	***OVA antigen /footpad***
Type 1	**TMX**	Sigma-Aldrich T2684	Water-in-oil emulsion (1:1)	30 µg	80 µg
	SAS	Sigma-Aldrich S6322-1VL	Oil-in-water-emulsion (1:1)	30 µg	80 µg
	CpG ODNs	Invivogen vac-1826-1	Aqueous (saline) solution	30 µg	80 µg
	Montanide ISA 720 VG	SEPPIC	Water-in-oil emulsion (1:1)	30 µg	80 µg
Type 2	**Alum**	Serva 12261.01	Water-in-oil emulsion (1:1)	-----	80 µg
	IFA	Sigma-Aldrich F5506-10X10ML	Water-in-oil emulsion (1:1)	-----	80 µg

5. Flow cytometry analysis

Eleven days after immunization (Day 11), draining LNs (popliteal and inguinal LNs) from B6 and Balb/c mice, were harvested and mashed through a 70µm mesh into PBS 1X, 2% (v/v) of Foetal calf serum (FCS). For identification of OVA-specific Tfh cells and GC B cells, $1x10^6$ cells were initially incubated with purified anti-CD16/32 to block unspecific binding of antibodies to Fc receptors, and then labelled with anti-CXCR5-biotin (15min at 37ºC and 15min at RT).

After washing (300g, 5min, 4ºC), cells were labelled with anti-DO11.10 (Balb/c mice), anti-$V_\beta5$ and anti-$V_\alpha2$ (B6 mice), anti-Fas, anti-PD-1, streptavidin, anti-GL-7, anti-CD25, anti-CD44, anti-CD19, anti-CD4, anti-Thy1.2, anti-Thy1.1 and anti-CD3 (30min, 4ºC) (Table 7.1 of attachments). Whenever possible, exclusion of dead cells was performed using a Viability dye (Life Technologies, Cat. No. L34957). Stainings were performed in PBS 1X, 2% (v/v) FCS. All samples were analysed using a LSR Fortessa (BD Biosciences) and the results processed with the FlowJo software version 8.7.1 (Tree Star Inc).

5.1 OVA-specific Tfh cell sorting

For sorting of antigen specific-Tfh and antigen specific-Th cells, $CD4^+$ T cells from each mice were processed individually and first enriched using 10µl/10^7cells of CD4 MicroBeads (L3T4) (Miltenyi Biotec-Citomed, Cat. No.130-049-201). Then, the positively selected cells were subsequently isolated using MACS Separation Columns as described by the manufacturer (Miltenyi Biotec-Citomed, Cat. No.130-042-401).

OVA-specific Tfh cells ($CD4^+CD44^+$ $CXCR5^+PD\text{-}1^+$ $Thy1.2^+$ $DO11.10^+$/ $V_\beta5^+V_\alpha2^+$) and OVA-specific activated-Th cells ($CD4^+CD44^+$ $CXCR5^-PD\text{-}1^-$ $Thy1.2^+DO11.10^+$/ $V_\beta5^+V_\alpha2^+$) were collected to lysis buffer (3,5µl of 0,2% (v/v) Triton X-100, 2U/µl RNAse inhibitor in H_2O), using BD FACSAria IIu, and frozen at -80ºC.

6. Th1 and Th2 polarization *in vitro*

6.1 Generation of mouse bone marrow-derived Dendritic Cells

Bone marrow-derived dendritic cells (BM-DC) were generated from B6 mice. The femurs were separated from muscle tissue, briefly cleaned in 70% (v/v) ethanol and washed with complete RPMI (RPMI 1X supplemented with 10% (v/v) heat inactivated FCS, 10mM HEPES, 50µM 2-mercaptoethanol, 100U/ml penicillin/ 100µg/mL Streptomycin, 2mM glutamine and 100nM sodium pyruvate- cRPMI). The ends of the bones were cut and a 27-gauge needle fitted into a syringe was used to flush the BM out with cRPMI. Following homogenization, erythrocytes were lysed by incubation (5min, RT) in Ammonium-Chloride-Potassium (ACK) lysing buffer (8,024mg/l of NH_4Cl, 1,001mg/l $KHCO_3$ and 3.722 mg/l $EDTA.Na_2 \cdot 2H_2O$). Cells were washed in PBS 1X, 2% (v/v) FCS and cultured, in sterile plate without tissue culture treatment, at $1x10^6$ cells/ml in cRPMI media supplemented with 20ng/ml recombinant murine Granulocyte macrophage colony-stimulating factor (GM-CSF) (PeproTech, Cat. No.315-03). The medium was changed every 3 days (Day 3 and Day 6). DCs were harvested on day 7 and co-cultured with naïve $CD4^+$ T cells, as indicated below.

6.2 Priming of naïve T cells

Naïve $CD4^+$ T cells were purified and enriched from spleen, mesenteric and inguinal LNs of OT-II $Rag^{-/-}$ mice. Red blood cells were eliminated using ACK lysis method and $CD4^+$ T cells positively selected using CD4 (L3T4) MicroBeads, as described above.

Naïve T cells ($3x10^4$ cells) were cultured at a ratio of 2:1 with allogeneic DCs in 96-well flat-bottom plates (Costar, Corning, NY, USA). T-DC co-culture was maintained for 4 days at 37ºC and 5% CO_2 in the conditions presented in table 3.4. All cells were cultured with DCs, OVA antigen and IL-2 as a basal stimulation. For Th1 polarization, anti-IL-4 and IL-12 were added, whereas anti-IFN-γ and IL-4 were used for Th2 polarization.

As a negative control (non-polarized cells), naïve $CD4^+$ T cells were cultured just with DCs and OVA. As a positive control (plate-bound), naïve $CD4^+$ T cells received activation signals from anti-CD3 and ani-CD28, as DCs were not used.

Table 3.4- Culture conditions for differentiation of naïve $CD4^+$ T cells.

Condition	*Reagent*	*Stock*	*Work concentration*	*Vendor Cat. No.*
Basal stimulation	**Ova**lbumin	500 µM/ml	10 µM /ml	Sigma, Grade 5503
	Mouse **IL-2** Recombinant Protein	100 µl/ml	5 ng/ml	eBioscience 14-8021-64
Th1 condition	Anti-mouse **IL-4** Purified BVD6-24G2	0,5 mg/ml	10 µg/ml	PeproTech 214-14
	Murine **IL-12**	100 µl/ml	10 ng/ml	PeproTech 210-12-B
Th2 condition	Anti-mouse **IFN-γ** Purified R4-6A2	1 mg/ml	10 ng/ml	LabClinics 16-7312-85
	Recombinant Murine **IL-4**	100 µl/ml	10 ng/ml	LabClinics 14-7042-85
Plate bound	Anti-Mouse **CD3**e Functional Grade Purified	1 mg/ml	3 µg/ml	eBioscience 16-0031-86
	Anti-Mouse **CD28** Purified	500 µl/ml	2 µl/ml	eBioscience 14-0281-86

The primed Th1 and Th2 cells were stimulated with phorbol-12-myristate-13-acetate (PMA), Ionomycin, BrefeldinA (BFA) and Golgi stop in cRPMI (4h, 37ºC and 5% CO_2).

Table 3.5- Stimulation conditions for promotion of cytokine production.

Reagent	*Stock concentration*	*Work concentration*
PMA	2,5 mg/ml	50 ng/ml
Ionomycin	2,5 mg/ml	500 ng/ml
BFA	2,5 mg/ml	5 µg/ml
Golgi Stop	1500x	1x [0,66 µl/ml]

6.3 Cell surface and intracellular staining

Cultured cells were incubated with purified anti-CD16/32 (15min, 4ºC) and then surface stained with anti-CD25, anti-CD4 and Livedead (30min, 4ºC). Cells were washed with PBS 1X, 2% (v/v) FCS, BFA and Golgi stop and fixed with Direct Fixation (eBioscience, Cat. No.88-8823-88) or 1:3- Fix/Perm concentrate: Fix/Perm diluent (eBioscience, Cat. No.00-5521-00) for cells that will be stained for cytokines or transcription factors, respectively (30min, on ice).

Then, after centrifugation (300g, 3min, 4ºC), cells were permeabilized with Perm Buffer (eBioscience, Cat. No.00-5521-00) and stained with anti-IFN-γ, anti-IL-13, anti-IL-4, anti-IL-21, anti-Gata-3, anti-T-bet and anti-Bcl-6 diluted in Perm Buffer. Cells were washed (PBS 1X, 2% (v/v) FCS) and data collected using the LSR Fortessa flow cytometer (BD Biosciences) and analysed using FlowJo software version 8.7.1 (Tree Star Inc).

7. Real-time quantitative-PCR (RT-qPCR)

Sorted cells ($5x10^4$ cells), namely Tfh cells ($CD4^+CD44^+$ $CXCR5^+PD\text{-}1^+$), activated-Th cells ($CD4^+CD44^+CXCR5^-PD\text{-}1^-$), non-activated Th cells ($CD4^+CD44^-$ $CXCR5^-PD\text{-}1^-$), B cells ($CD19^+$ $FAS^-GL\text{-}7^-$) and GC B cells ($CD19^+$ $FAS^+GL\text{-}7^+$) were collected from naïve mice and from immunized mice with type 1 or type 2 adjuvants.

7.1 RNA extraction from T cells

Cells were lysed in 350µl of lysis buffer (buffer RLT, RN Easy MiniKit, Qiagen) with 10µl/ml β-mercaptoethanol and homogenized by vortexing. For RNA extraction, the Quick-StartProtocol provided by RNeasy® Mini Kit (QUIAGEN) was followed.

7.2 cDNA synthesis from total RNA

cDNA was synthesized from total RNA (11µl) by reverse transcription using Oligo(dT) and SuperScript II Reverse Transcriptase following the protocol available from Invitrogen and partially available on table 3.6. cDNA synthesis was performed using a PCR thermal cycler: 65ºC 5min, quick chill on ice, 42ºC 2min, 42ºC 50min and 70ºC 15min.

Table 3.6- 1x SuperScript II reverse transcriptase master mix.

Reagent	***Volume 1x master mix (µl)***
Oligo(dT)$_{12-18}$ (500 µg/mL)	1
dNTP Mix (10 mM each)	1
RNA template	11
5x First-Strand buffer	4
DTT (0.1 M)	1
RNaseOUT™ Recombinant RNase Inhibitor (40 units/µl)	1
SuperScript^{TM} II (200 units)	1
	20

7.3 Quantitative Real time PCR

Quantitative Real time polymerase chain reaction (qPCR) was performed using primers, specific for CXCR5, Bcl-6, Gata-3, T-bet, IL-4 and IFN-γ genes (Table 3.8), using Power SYBR® Green PCR Master Mix (Life Technologies) to detect the amplified PCR product from synthetized cDNA. Primers amplifying a segment of the housekeeping gene encoding β-2-microglobulin, since it is constitutively expressed by most cells, and in relation to which relative quantification will be performed. Reaction was processed in the RT-PCR Viia 7 System machine, following programed PCR thermal cycler: 50ºC 2min, 95°C 10min, 95ºC 15sec, 60 °C 1min (40-45 cycles); Melt curve, 95ºC 15sec, 65°C 1min, 95ºC 15sec. Relative expression was calculated using the $2^{-\Delta Ct}$ method.

Table 3.7- RT-qPCR master mix reaction.

Reagent	*Work concentration*	*Volume 1x master mix (µl)*
Template	1:5 dilution	2
SYBR® Green (1x)	1x	4
Primer F (10 nM)	100 nM	0,08
Primer R (10 nM)	100 nM	0,08
H_2O	---	1,84

Table 3.8- Primers used in analysis of Tfh, activated-Th and non-activated-Th cells, B cells and GC B cells.

Primers (Target gene)	*Sequence*	*Orientation*
CXCR5	GAG CTG CAG CTA TGA ACT AC	Forward
	AGG AGG AAG ATG AGG CTG TA	Reverse
Bcl-6	AGT GAG AGT CAC TCA CCA CT	Forward
	GCA GTC ACA TTC GTT GCA GA	Reverse
Gata-3	CAC TAC CTT TGC AAT GCC TG	Forward
	AGC TTG TAG TAC AGC CCA CA	Reverse
T-bet	GGA AGC TAA AGC TCA CCA AC	Forward
	AGC TGA GTG ATC TCT GCG TT	Reverse
IL-4	CAT ATC CAC GGA TGC GAC AA	Forward
	TCT TCA AGC ATG GAG TTT TC	Reverse
IFN-γ	ACT GGC AAA AGG ATG GTG AC	Forward
	GTG GGT TGT TGA CCT CAA AC	Reverse
β-2-	CTG CAG AGT TAA GCA TGC CAG TAT	Forward
microglobulin	ATC ACA TGT CTC GAT CCC AGT AGA	Reverse

4. Results

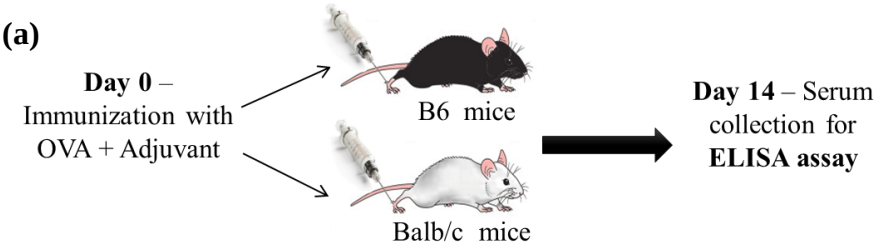
1. CpG ODNs was the best adjuvant to induce Th1 responses, whereas Incomplete Freund's Adjuvant (IFA) was better at inducing Th2 responses

With the purpose of dissecting the phenotype of antigen-specific Tfh cells that are generated after immunization with OVA protein in conjunction with either type 1 or type 2 adjuvants, it was essential to optimize the ideal immunizing conditions.

In these experiments, 6-8 weeks old mice were immunized in the footpad with each of the adjuvants described and as indicated in scheme 4.1. Serum was collected on day 14 in order to analyse the humoral response induced by each adjuvant using ELISA assay.

Scheme 4.1- Experimental design of the immunization assay of B6 and Balb/c mice. (a) On day 0, mice were immunized with 80µg of OVA along with the indicated type 1 or type 2 adjuvants (listed in **(b)**). On day 14, serum was recovered from peripheral blood and used to determine antibody titres by ELISA.

(a)



(b)

	Adjuvant	*CpG ODNs /footpad*	*OVA antigen /footpad*
Type 1	**TMX**	30 µg	80 µg
	SAS	30 µg	80 µg
	CpG ODNs	30 µg	80 µg
	Montanide ISA 720 VG	30 µg	80 µg
Type 2	**Alum**	-----	80 µg
	IFA	-----	80 µg

To investigate Th1 responses, several type 1 adjuvants were tested in Balb/c and B6mice. More specifically, CpG ODNs only or in combination with TMX and SAS were tested in both strains, while Montanide ISA 720 VG was tested only in B6 mice.

Synthetic oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODNs) are normally present in bacterial DNA at a 20-fold greater frequency compared to mammalian DNA. CpG ODNs are recognized by TLR-9, which induces the activation of DCs and Th1 cells, that, in turn, secrete typical pro-inflammatory cytokines, such as TNF-α, IFN-γ, IFN-α and IL-12 (58). In this project a mouse specific type B CpG ODNs was used as an efficient inducer of Th1 immunity, with predominant production of IgG2a.

TMX is a stable water-in-oil emulsion that, upon subcutaneous administration, forms a depot effect with slow release of the antigen, stimulating cellular and humoral immune responses. It is characterized by the production of high antibody titres, mainly IgG2a, even without boost immunization (59).

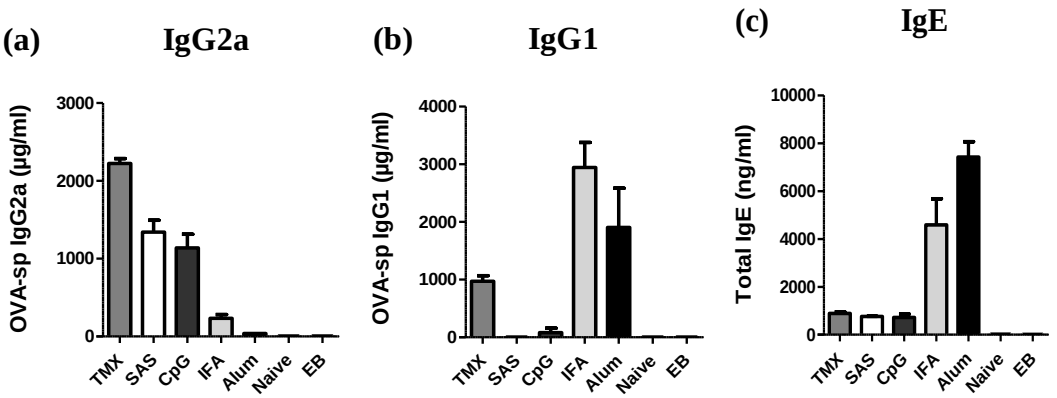
SAS is a stable oil-in-water emulsion which contains Monophosphoryl lipid A (MPLA), an immunostimulating TLR-4 agonist formed by detoxified lipopolysaccharide (LPS) from *Salmonella minnesota R595*. MPLA is considerably less toxic than LPS, whilst maintaining immunostimulatory activity, inducing a strong Th1 response (60).

Montanide ISA 720 VG, tested only in B6 mice, is also a water-in-oil emulsion that promotes the formation of a depot at the site of injection (61). It has been shown that it induces both Th1 and Th2 responses, as antigen-specific IgG1 and IgG2c titres were observed in response to vaccine consisting of *Schistosoma mansoni* cathepsin B formulated in Montanide ISA 720 VG (62).

As for type 2 adjuvants, IFA and Alum were tested in both strains and in Balb/c mice only, respectively. IFA induces a predominantly Th2-biased response through the formation of a depot at the injection site, enabling the slow release of antigen and the stimulation of high and long-lasting antibody producing plasma cells (63). This adjuvant efficiently induced the production of high titres of IgG1 and also of IgE.

Alum adjuvant stimulate the immune system in several ways, including the formation of a depot at the injection site, thereby extending the time for antigen capture by the APCs and its presentation to lymphocytes. Additionally, this adjuvant can increase the production of Th2 cytokines, IL-4, IL-5 and IL-10, which stimulate humoral responses with class-switch and production of IgG1and IgE (56,64).

Balb/c mice



B6 mice

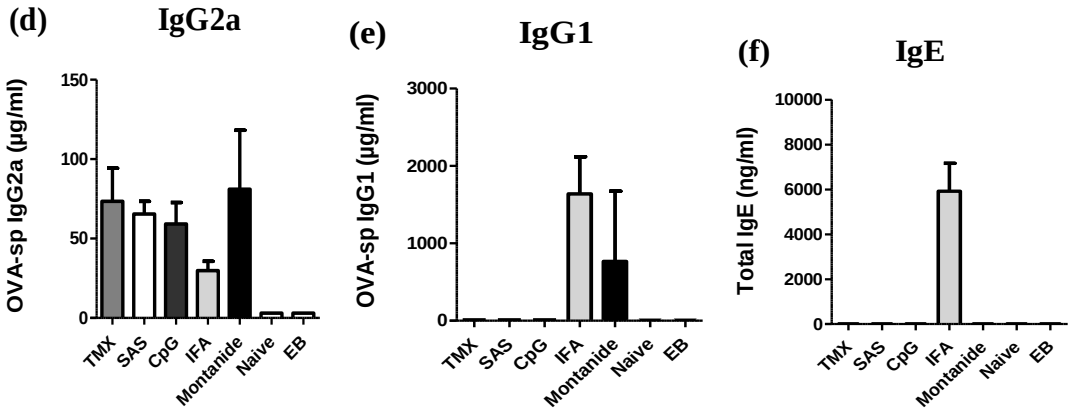


Figure 4.1- Titres of IgG2a, IgG1 and IgE measured on day 14 in the serum of Balb/c and B6 mice immunized with OVA and selected adjuvants. (a, d) OVA-specific IgG2a, **(b, e)** OVA-specific IgG1 and **(c, f)** Total IgE titres analysed in mice serum (indicated as concentration at µg/ml for IgG and ng/ml for IgE). **(a-c)** Ig measured in Balb/c mice immunized with TMX (n=4), SAS (n=4), CpG ODNs (n=4), IFA (n=4), Alum (n=4) and non-immunized mice, designed as naïve (n=2); **(d-f)** Ig measured in B6 mice immunized with TMX (n=6), SAS (n=6), CpG ODNs (n=6), Montanide (n=6), IFA (n=6) and naïve mice (n=2); This experiment was performed three times and each sample tested in duplicates.

The best adjuvant was chosen considering a clear production of characteristic Th1 (IgG2a) and Th2 antibodies (IgG1 and IgE).

TMX conjugated with 30µg of CpG ODNs seemed to be the best adjuvant for the generation of Th1 responses, because it promoted the production of high levels of IgG2a in both mouse strains (Figures 4.1a and 4.1d). This adjuvant promoted the strongest and cleanest Th1 response in B6 mice, although it also stimulated the production of considerable titres of IgG1 in Balb/c mice (Figure 4.1b). Considering that the same adjuvant had to be used in both mouse strain to establish a parallel between the immune responses, TMX was excluded as a good Th1 inducer.

Mice were also immunized with SAS conjugated with 30µg of CpG ODNs and with CpG ODNs (only) and as can be seen similar titres of IgG2a were produced (Figures 4.1a and 4.1d). Thus, the effect of SAS was apparently not very relevant for the induction of a good Th1 response. The use of CpG ODNs led to an immune response with a typical Th1 profile, mostly characterized by elevated IgG2a levels and low IgG1 levels (Figures 4.1a-b and 4.1d-e). Furthermore, and relevantly for practical and of animal welfare reasons, the use of CpG by itself was also preferred because it is cheaper and easier to prepare, and promotes less animal suffering, as no swelling can be detected in the injected paws.

Montanide ISA 710 VG was only tested in B6 mice and was considered an unsuitable Th1 adjuvant because it induced the production of high levels of both IgG2a and IgG1 (Figures 4.1d and 4.1e).

IFA was the most efficient adjuvant to polarize for Th2 responses either in Balb/c or in B6 mice, as it induced high titres of IgG1 and IgE (Figures 4.1b-d and 4.1c-e, respectively), with low production of IgG2a (Figure 4.1a-c). Alum was tested in Balb/c mice and it promoted lower IgG1 production (Figure 4.1b), when compared with IFA, paired with a huge IgE class-switch (Figure 4.1c).

Summarizing, ideal immunization conditions to drive Th1 (OVA-CpG ODNs) and Th2 (OVA-IFA) responses have been established, based on the skewed production of either IgG2a or IgG1/IgE, respectively. Thus, we used these conditions in subsequent experiments.

2. Phenotypic characterization and recovery of Tfh cells

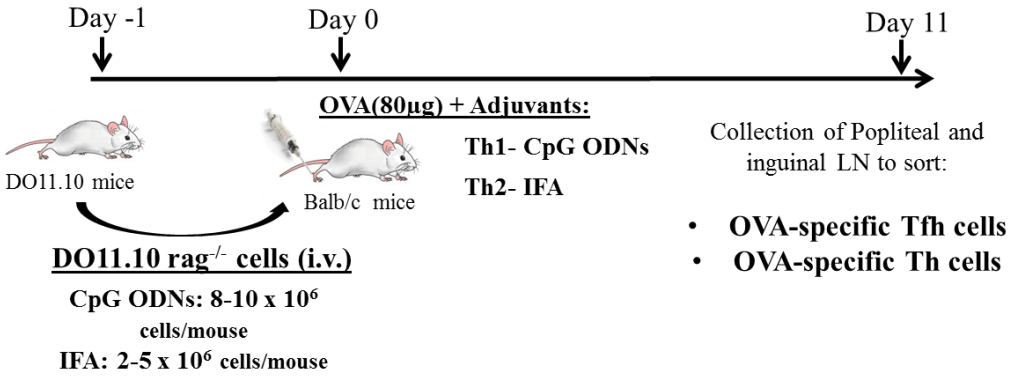
It was then essential to investigate and identify the phenotype of Tfh and antigen-specific Tfh cells, to ensure successful recovery, in order to dissect their molecular signature via RNA-sequencing analysis. Adoptive cell transfers from transgenic mice into immunocompetent mice was the chosen approach to allow identification, tracking and recovery of antigen-specific Tfh cells.

In these experiments, cells collected from spleens and LNs of DO11.10 $Rag^{-/-}$ (Balb/c background) and OT-II $Rag^{-/-}$ (B6 background) donor mice were i.v transferred into Balb/c and B6Thy1.1 or B6Thy1.1/Thy1.2 recipients mice, respectively.
DO11.10 $Rag^{-/-}$ and OT-II $Rag^{-/-}$ mice express a transgenic TCR that specifically recognizes chicken ovalbumin (OVA) peptide (323-339) presented by MHC class II molecule H2-Ab1 (I-Ab). These mice are homozygous for the transgene and deficient in the recombinase activating gene 2 (Rag2 gene) and, therefore, are not able to develop endogenous mature T or B cells. Importantly, all these T cells are identic in having the same OVA-specific TCR.

OT-II $Rag^{-/-}$ are transgenic mice that express $V_{\alpha}2$ and $V_{\beta}5$ rearrangement of the α and β-chains, respectively, of the TCR. Moreover, Thy-1 or CD90 molecule is a conserved cell surface protein, formed by two distinct alleles, Thy1.1 (CD90.1) and Thy1.2 (CD90.2) that differ only in one amino acid at position 108 of the polypeptide chain. Thy-1 can be used as a congenic marker for T cell identification since Thy1.2 is expressed by most mouse strains, including Balb/c, B6 mice, and also in transgenic OT-II $Rag^{-/-}$ and DO11.10 $Rag^{-/-}$ mice, whereas Thy1.1 expression can be detected in B6 Thy1.1 or B6 Thy1.1/Thy1.2 mice.

After cell transfer, recipient mice were immunized in the footpad combining OVA and the selected adjuvants, CpG ODNs for Th1 and IFA for Th2 priming. On day 11, draining LNs, namely popliteal and inguinal LNs, were collected (Scheme 4.2). As previously demonstrated in kinetic studies done in the host laboratory (65), the peak of the GC reaction is around day 11, since at day 12 the density of T cells within the GC is already decreasing. As maximum recovery of cells is one of our goals, day 11 was therefore, chosen as the best for this study.

Scheme 4.2- Experimental design of adoptive cell transfer and immunization assay. On day -1, cells from DO11.10 Rag$^{-/-}$ mice were transferred (i.v) into Balb/c mice. On day 0, mice were immunized in the hind footpad with 80µg of OVA conjugated with CpG ODNs (Th1) or IFA (Th2). On day 11, OVA-specific Tfh and OVA-specific Th cells were collected from draining LNs and sorted for further analysis. The same experimental design was performed in B6 mice, where cells from OT-II Rag$^{-/-}$ mice were transferred into B6Thy1.1/Thy1.2 or B6Thy1.1.



For phenotypical analysis, CD4$^+$ T cells were labelled with typical Tfh markers CXCR5 and PD-1, as well as with the activation markers CD25 and CD44. Moreover, the staining was complemented with anti-DO11.10 (KJ1-26) antibody (Balb/c mice), anti-V$_\beta$5 and V$_\alpha$2 (B6 mice) and anti-Thy1.2 and anti-Thy1.1 for tracking of the OVA-specific cells.

CD19$^+$ B cells were identified through expression of GL-7 and FAS (CD95) known as typical markers expressed by B cells that are inside the GC.

2.1 Tfh cells were phenotypically characterized as CD4$^+$ CXCR5$^+$PD-1$^+$ while GC B cells were identified as CD19$^+$GL-7$^+$FAS$^+$

Tfh cells, a subset of CD4$^+$ T cells, were identified by simultaneous expression of CXCR5 and PD-1 as CD4$^+$CXCR5$^+$PD-1$^+$ (Figure 4.2d), while the remaining CD4$^+$CXCR5$^-$PD-1$^-$ cells were considered as extra follicular Th cells (Figure 4.2d). OVA-specific Tfh cells from Balb/c mice were recovered as CD4$^+$CXCR5$^+$PD-1$^+$Thy1.2$^+$DO11.10$^+$, since the transgenic TCR can be identified with the specific DO11.10 antibody (Figures 4.2e, h).

OVA-specific Tfh cells from B6 mice were identified as $CD4^+$ $CXCR5^+PD\text{-}1^+$ $Thy1.2^+Thy1.1^-$ $V_\beta5^+V_\alpha2^+$ (Figure 4.3). The same Tfh cell phenotype was observed in both B6 and Balb/c backgrounds, either when mice were immunized with CpG ODNs or IFA adjuvants.

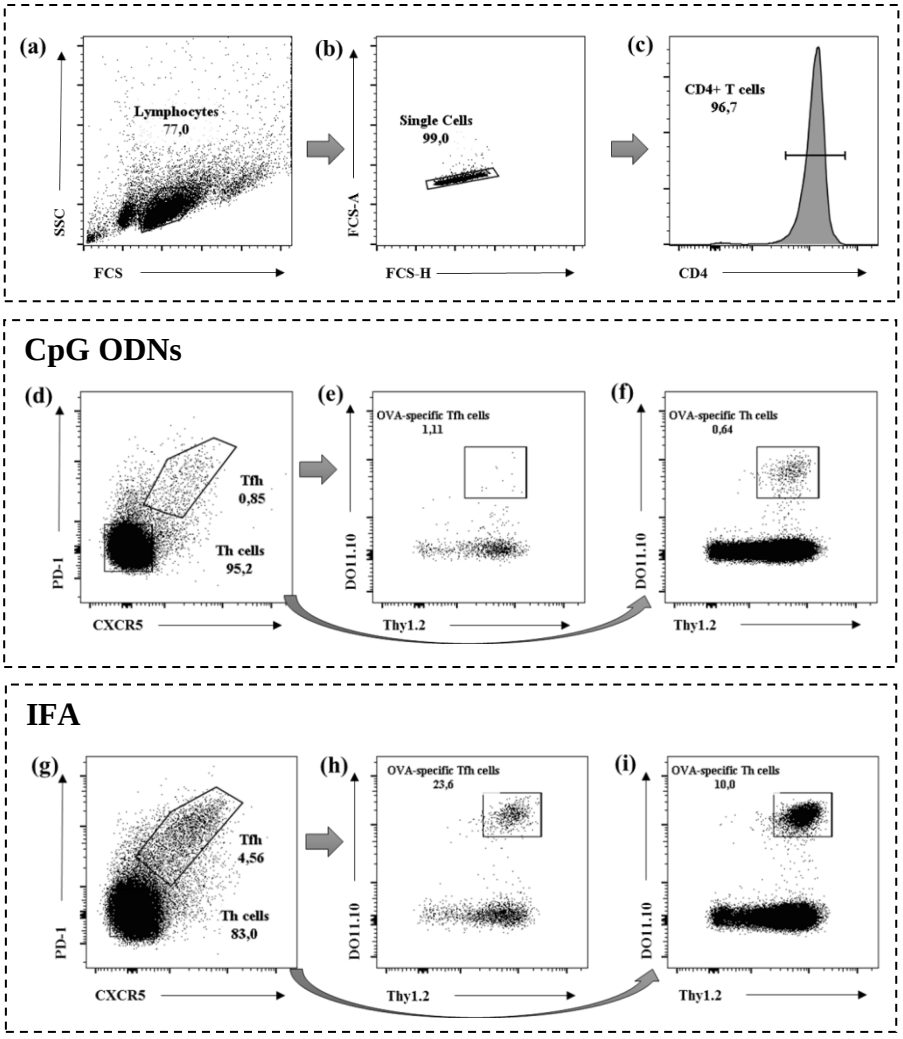


Figure 4.2- Gating strategy for OVA-specific Tfh cells identification, from draining LNs of Balb/c mice immunized with OVA-CpG ODNs and OVA-IFA. **(a)** Lymphocytes population **(b)** Doublet cells exclusion **(c)** $CD4^+$ T population; **(a-c)** gating strategy is the same for both adjuvants; **(d, g)** Tfh cells ($CXCR5^+PD\text{-}1^+$) and Th cells outside the GC ($CXCR5^-PD\text{-}1^-$) **(e, h)** OVA-specific Tfh cells ($Thy1.2^+DO11.10^+$) and **(f, i)** OVA-specific Th cells ($Thy1.2^+$ $DO11.10^+$). **(d-f)** results from mice immunized with OVA-CpG ODNs (n=12); **(g-i)** results from mice immunized with OVA-IFA (n=8). This experiment was performed four times.

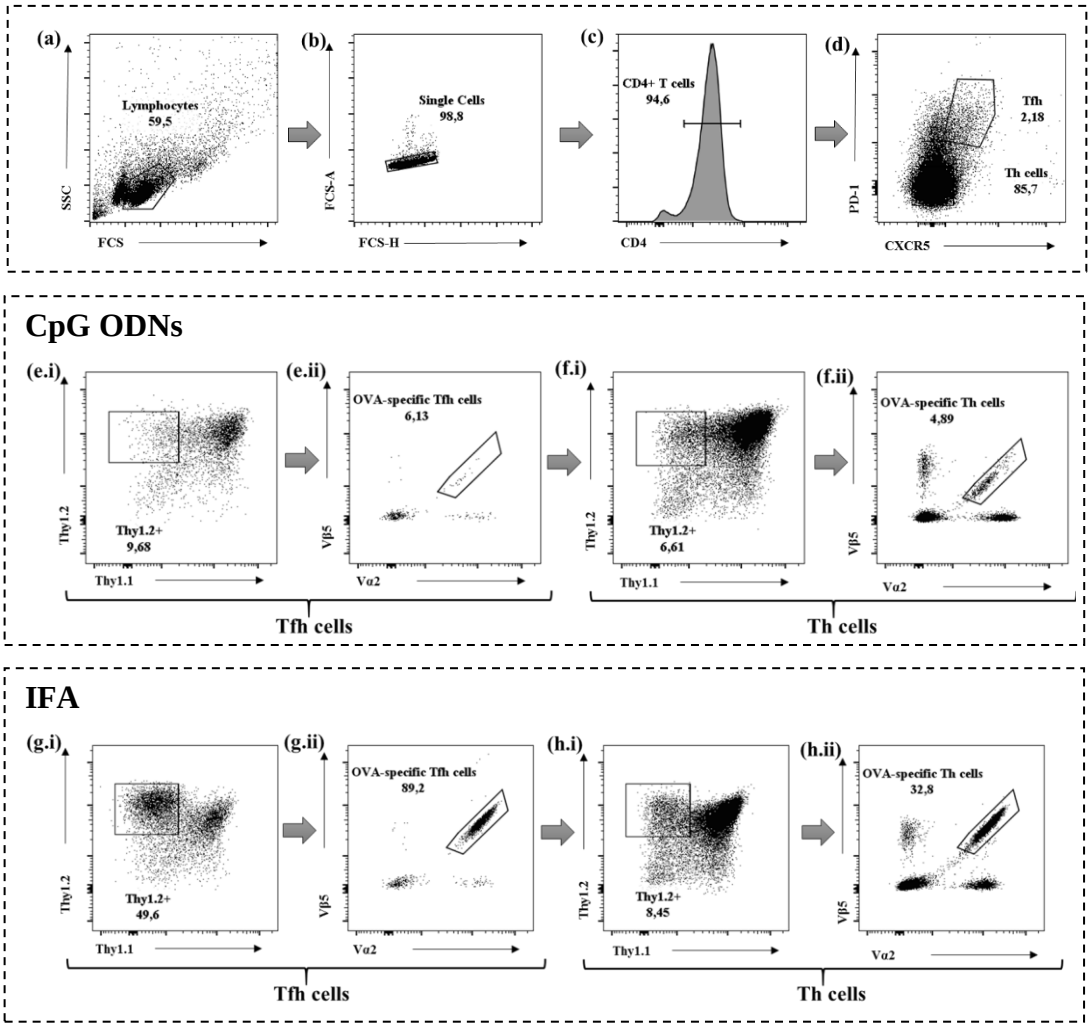


Figure 4.3- Gating strategy for OVA-specific Tfh cells identification, from draining LNs of B6 mice immunized with OVA-CpG ODNs and OVA-IFA. **(a)** Lymphocytes population **(b)** Doublet cells exclusion **(c)** $CD4^+$ T population **(d)** Tfh cells ($CXCR5^+PD\text{-}1^+$) and Th cells outside of GC ($CXCR5^-PD\text{-}1^-$) **(e, g)** OVA-specific Tfh cells, **(e.i, g.i)** $Thy1.2^+Thy1.1^-$ and **(e.ii, g.ii)** $V_\beta5^+V_\alpha2^+$; **(f, h)** OVA-specific Th cells, **(f.i, h.i)** $Thy1.2^+Thy1.1^-$ and **(f.ii, h.ii)** $V_\beta5^+V_\alpha2^+$. **(e-f)** results from mice immunized with OVA-CpG ODNs (n=10); **(g-h)** results from mice immunized with OVA-IFA (n=6). This experiment was performed twice.

B cells that received help from Tfh cells were also investigated and the gating strategy for GC B cell identification is shown in figure 4.4a-d. Two distinct $CD19^+$ populations were identified (Figure 4.4d), one population was characterized by expression of high levels of GL-7 and FAS (GC B cells), and a second population outside the GC designated simply as B cells ($CD19^+GL\text{-}7^-FAS^-$).

Moreover, higher percentages of B cells were mobilized into the GC (GC B cells) of Balb/c mice immunized with OVA-IFA, rather than with OVA-CpG ODNs (Figures 4.4f and 4.4e, respectively). Similarly, higher numbers of total $CD19^+$ B cells were collected from B6 mice immunized with OVA-IFA, but low percentages were observed for GC B cells ($CD19^+GL\text{-}7^+FAS^+$).

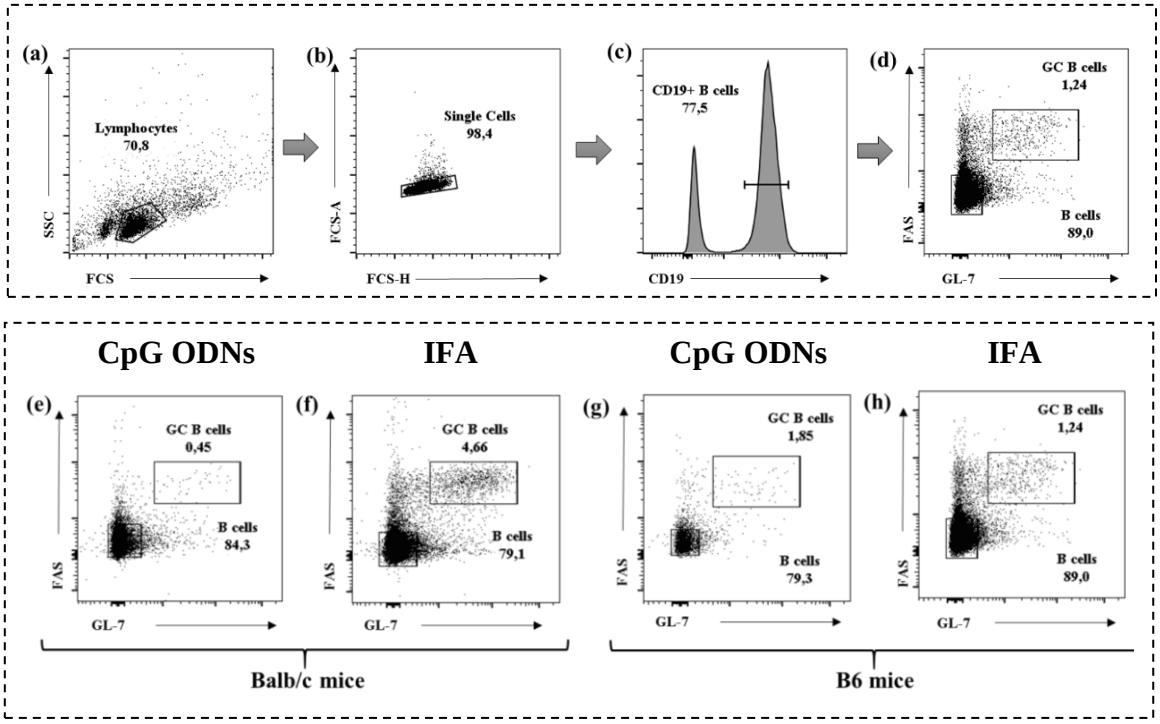


Figure 4.4- Gating strategy for GC B cells identification from draining LNs of Balb/c and B6 mice immunized with both type 1 and type 2 adjuvants. (a) Lymphocytes population **(b)** Doublet cells exclusion **(c)** $CD4^+$ T population; **(a-c)** gating strategy is the same for both adjuvants; **(e, f)** Balb/c mice were immunized with OVA-CpG ODNs (n=12) and OVA-IFA (n=8), respectively; **(g, h)** B6 mice were immunized with OVA-CpG ODNs (n=10) and OVA-IFA (n=6), respectively; These results were analysed using the Tfh cell analysis strategy previously shown.

2.2 Tfh cells express high levels of the activation marker CD44, while expressing no CD25

Activation profiles were also investigated in order to define and collect activated-Th cells that were participating in the response to OVA antigen. There are few important activation markers expressed on T cells. CD44 is a marker of T cell activation and a property of long-lived memory cells that are implicated in cell migration, activation and differentiation (66,67). It is the classical marker of activation because it remains expressed almost indefinitely once the cell is activated (68). CD25 is a high-affinity IL-2 receptor α chain, rapidly upregulated by antigen-specific T cells after TCR stimulation. So, it is upregulated in the early days of the response, but then tends to go back down with decreasing IL-2 availability (68,69).

Therefore, $CD4^+$T cells collected from draining LNs, as described above, were also stained with anti-CD44 and anti-CD25. The results obtained from Balb/c mice immunized with CpG ODNs-OVA are displayed as representative of both immunizing conditions (Th1 and Th2) and also of the results obtained with the B6 mice, as expression of CD25 and CD44 was analogous (Figure 4.5). The gating strategy was the same as the one presented on figure 4.2.

Tfh cells are referred in the literature as $CD25^-$, which makes sense because IL-2 signalling via STAT-5 inhibits Tfh cell differentiation (68,69). Nevertheless, CD44 expression has been widely studied in T cells, but not in Tfh cells. In this work, the activation status of Tfh cells ($CD4^+CXCR5^+PD\text{-}1^+$) was defined as $CD25^-$ and $CD44^{high}$, as observed in figure 4.5a and figure 4.6b.

Activation markers on extra-follicular Th cells ($CD4^+CXCR5^-PD\text{-}1^-$) were also investigated. In figure 4.5b.i, two distinct populations are visible, Th cells that are $CD25^+$ and Th cells that are $CD25^-$. Additionally, $CD44^+$ Th cells and $CD44^-$ Th cells were detected (Figure 4.5b.ii), which from now will be designated as activated-Th cells ($CD4^+CXCR5^-PD\text{-}1^-CD44^+$) and as non-activated-Th cells ($CD4^+CXCR5^-PD\text{-}1^-CD44^-$), irrespective of CD25 expression. In figure 4.6a the relative expression of CD44 on Tfh (solid red line), activated-Th (dashed blue line) and non-activated-Th cells (solid black line), revealed that Tfh cells and activated-Th cells expressed similar levels of CD44.

Additionally, OVA-specific Tfh cells ($CD4^+CXCR5^+PD\text{-}1^+DO11.10^+$) were identified as $CD25^-CD44^+$ (Figure 4.5a), while OVA-specific Th cells ($CD4^+CXCR5^-PD\text{-}1^-DO11.10^+$) were mostly $CD25^-CD44^+$, despite some cells that express low levels of CD44 (Figure 4.5b).

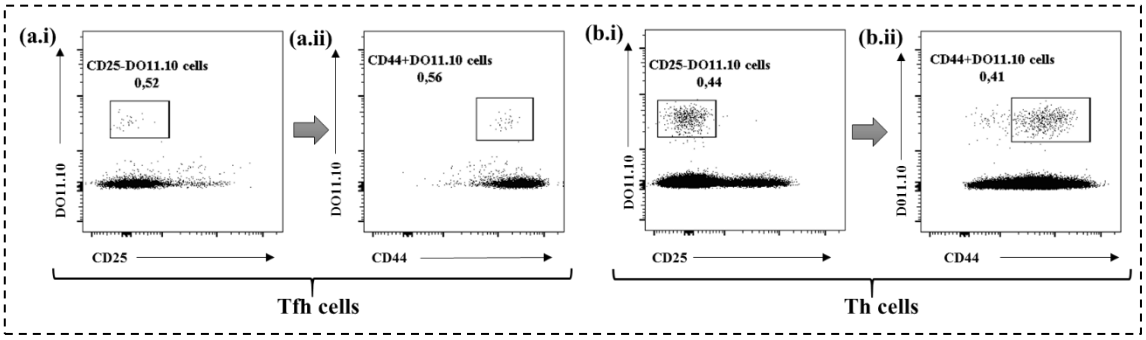


Figure 4.5- Expression of CD44 and CD25 on OVA-specific Tfh cells and OVA-specific Th cells. (a.i) CD25 expression on OVA-specific Tfh cells ($DO11.10^+$); **(a.ii)** CD44 expression in OVA-specific Tfh cells ($DO11.10^+$); **(b.i)** CD25 expression on OVA-specific Th cells ($DO11.10^+$); **(b.ii)** CD44 expression in OVA-specific Th cells. ($DO11.10^+$); Balb/c mice (n=4) were immunized with CpG ODNs-OVA and gating strategy are presented on figure 4.2. This experiment was performed twice.

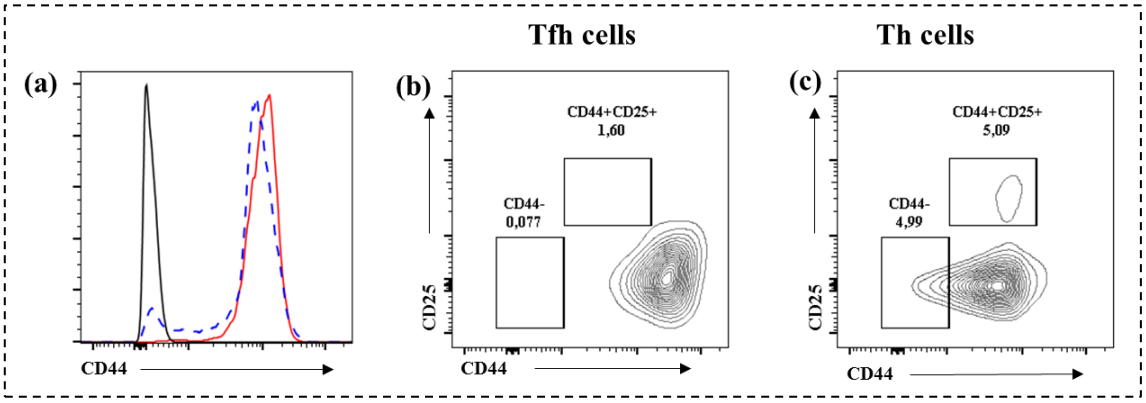


Figure 4.6- Comparative CD44 expression in Tfh cells and extra-follicular cells. (a) Tfh cells shown in solid red line, activated-Th cells ($CD4^+CXCR5^-PD\text{-}1^-CD44^+$) shown in dashed blue line and non-activated-Th cells ($CD4^+CXCR5^-PD\text{-}1^-CD44^-$) shown in solid black line; CD44 and CD25 expression in **(b)** Tfh cells ($CD4^+CXCR5^+PD\text{-}1^+$) and **(c)** extra-follicular cells ($CD4^+CXCR5^-PD\text{-}1^-$) from draining LNs of Balb/c mice (n=4) immunized with CpG ODNs-OVA. This experiment was performed twice.

2.3 IFA induces a better recruitment of $CD4^+$ T cells into GCs than does CpG ODNs

In both mouse strains, cell recruitment into the GC was significantly different, when mice were immunized either with CpG ODNs or IFA adjuvants. When OVA-CpG ODNs were injected as an aqueous solution of OVA and TLR-9 agonists, the promoted Th1 response displayed poor recruitment of $CD4^+$ T cells into the GC (Figures 4.2 and 4.3). Additionally, the majority of OVA-specific T cells remained outside the GC (Table 4.1).

On the contrary, IFA is an emulsion providing slow release of OVA antigen that can extend the effect on the immune system, promoting better mobilization of OVA-specific cells into the GC (Figures 4.2 and 4.3). There, cells can continue to provide help to GC B cells which, consequently, co-exist in higher numbers (Figure 4.4).

Similar percentages (1-2%) of Tfh cells in both Balb/c and B6 mice were found, when immunizing with OVA-CpG ODNs (Figures 4.2d and 4.3d). However, higher absolute numbers of OVA-specific cells were mobilized into GCs in B6 mice comparing with Balb/c mice, as visible on table 4.1. Worthy of note is the fact that in mice stimulated with OVA-CpG ODNs the majority of OVA-specific cells remained outside of GC.

In Balb/c and B6 mice stimulated with OVA-IFA, between 3,5-5% of the total $CD4^+$ T cells were mobilized into the GC in response to OVA antigen. Higher numbers of OVA-specific cells were found in the GCs of B6 mice compared to Balb/c mice (Table 4.1).

Furthermore, IFA induced a better recruitment of OVA-specific cells into GCs, comparing with CpG ODNs. The same results were obtained when sorting OVA-specific Tfh cells ($CD4^+CXCR5^+PD\text{-}1^+CD44^+Thy1.2^+$ $DO11.10^+/V_\beta5^+V_\alpha2^+$) and OVA-specific activated-Th cells ($CD4^+CXCR5^-PD\text{-}1^-CD44^+Thy1.2^+$ $DO11.10^+/V_\beta5^+V_\alpha2^+$) (Table 4.1). The indicated numbers of cells were recovered and collected to lysis buffer for subsequent RNA-sequencing analysis.

As cell recruitment was so different when mice were immunized with CpG ODNs or IFA adjuvants, it was necessary to optimize the number of transferred cells. In Balb/c mice, low numbers of OVA-specific cells were mobilized into the GC, comparing with B6 mice. So, in Balb/c mice, $2x10^6$cells and $4x10^6$cells were tested in immunization with OVA-IFA, since this adjuvant promoted notable cell recruitment into GC. Considering that cell recruitment under OVA- CpG ODNs immunization was even less efficient, $3x10^6$cells, $6x10^6$cells and $9x10^6$cells were tested.

Table 4.1- Number of OVA-specific Tfh and Th cells sorted on day 11 in B6 and Balb/c mice. Numbers displayed are representative of B6 and Balb/c mice that were analysed (n=6) under both conditions.

Adjuvant	*Cell Population (OVA-specific)*	*Number of cells (B6)*	*Number of cells (Balb/c)*
CpG ODNs	**Tfh**	450	58
	Th	500	150
	Tfh	2 400	155
	Th	4 700	292
IFA	**Tfh**	60 900	590
	Th	183 600	63
	Tfh	54 200	10 800
	Th	196 300	34 600

The number of sorted cells on day 11 (Table 4.2) revealed that in mice immunized with OVA-CpG ODNs, $3x10^6$ transferred cells were not sufficient to detect enough OVA-specific cells inside the GC. Cell recruitment was apparently similar when $6x10^6$ cells or $9x10^6$ cells were transferred into recipient mice. Taking this into consideration, it was decided to transfer 8-$10x10^6$cells to Balb/c and B6 mice immunized with CpG-ODNs. Under OVA-IFA stimulation, more cells could be recovered from Balb/c mice transferred with $2x10^6$ cells. However, when mice were transferred with $4x10^6$ cells there was also a significant number of OVA-specific cells in the GC (Table 4.2). Thus, it was decided to transfer between 2-$5x10^6$ cells to Balb/c and B6 mice immunized with OVA-IFA.

Table 4.2- Number of OVA-specific Tfh and Th cells sorted on day 11 in Balb/c mice. $3x10^6$, $6x10^6$ and $9x10^6$ cells were tested in mice immunized with CpG ODNs. $2x10^6$ and $4x10^6$ cells were injected in mice immunized with IFA.

Adjuvant	*Transferred cells*	*Population (OVA-specific)*	*Number of cells*
CpG ODNs	**$3x10^6$**	**Tfh**	63
		Th	12.600
	$6x10^6$	**Tfh**	500
		Th	8.000
	$9x10^6$	**Tfh**	500
		Th	25.300
IFA	**$2x10^6$**	**Tfh**	21.500
		Th	143.600
	$4x10^6$	**Tfh**	12.800
		Th	35.400

3. Molecular characterization of Tfh cells: Expression of transcription factors and cytokine production

The molecular characterization of the transcriptome of OVA-specific Tfh and OVA-specific Th cells collected from Balb/c and B6 mice immunized with OVA-CpG ODNs and OVA-IFA is currently being analysed by RNA-sequencing (GeneCore, EMBL).

In parallel, in a prelude attempt to begin dissecting their phenotype, an *ex vivo* analysis was performed on isolated Tfh cells via flow cytometry and RT-qPCR.

3.1 *in vit*ro polarized Th1 cells expressed T-bet and produced IFN-γ, while induced Th2 cells expressed Gata-3 and IL-13

An immune response can be identified through the expression of characteristic transcription factors, cell surface markers and also of given cytokines. Thus, it was important to confirm, by flow cytometry, whether all of these specific markers could be identified, when naïve $CD4^+$ T cells were induced with potent stimulators, *in vitro*, that polarized cells into a Th1 or a Th2 effector phenotype.

$CD4^+$ T cells require three different signals for their differentiation. The first signal is generated via TCR when DCs present the antigen (OVA) via MHC class-II, or alternatively by stimulation with an anti-CD3 antibody (1). The second signal is generated as a result of the interaction between the CD28 co-receptor, on the T cell, and the B7 family of co-stimulatory molecules on APCs. This signal can also be supplied with an anti-CD28 antibody (1). The third signal is provided by cytokines produced by the APCs or other cells (1).

Naïve T cells were cultured with DCs, OVA and IL-2 as a basal stimulation. For Th1 differentiation, anti-IL-4 and IL-12 were added, whereas anti-IFN-γ and IL-4 were used for Th2 polarization, incubating for 4 days at 37ºC and 5% CO_2. Then, induced Th1 and Th2 cells were stimulated with PMA and Ionomycin, that support T-cell activation, proliferation and cytokine production (70), and also with BFA and Golgi stop, which block secretory pathways, allowing intracellular cytokine accumulation (71).

As described in the literature, IL-12 stimulation induces the expression of the key transcription factor T-bet on Th1 cells, which consequently supports IFN-γ production (16). Expression of T-bet and IFN-γ were exclusively detected on naïve $CD4^+$ T cells under Th1 polarizing conditions (Figures 4.7a and 4.7c, respectively).

In contrast, Th2 cells produce several cytokines, namely IL-4, IL-5, and IL- 13, in response to the expression of the Gata-3 transcription factor (22,23). The presence of IL-4 and anti-IFN-γ (Th2 condition) induced Gata-3 stimulation and expression, which was not detected, either in non-polarized cells or cells under Th1 conditions (Figure 4.7b).

IL-13 could only be detected in cells under Th2 conditions (Figure 4.4d), even though at low levels when compared with the levels of IFN-γ produced by Th1 induced cells. Detection of IL-4 was attempted several times without success.

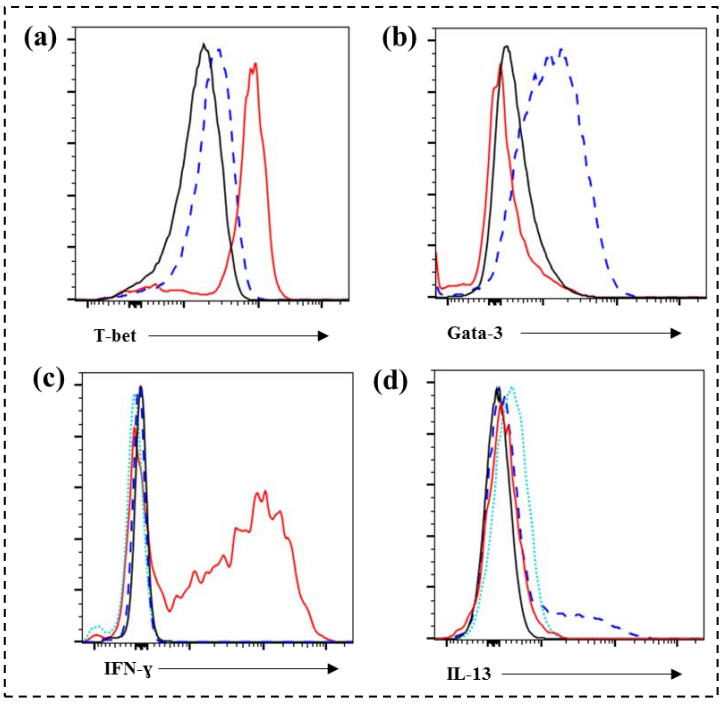


Figure 4.7- Expression of characteristic Th1 and Th2 transcription factors and cytokines on cells polarized *in vitro*. (a) T-bet was expressed on cells under Th1 condition (solid red line), comparing with non-polarized cells (solid black line) and Th2 cells (dashed blue line); **(b)** Gata-3 was expressed by Th2 cells (dashed blue line), while it was not detected in Th1 cells (solid red line) and in non-polarized cells (solid black line); **(c)** IFN-γ was produced by Th1 cells (solid red line) and **(d)** IL-13 was expressed by Th2 cells (dashed blue line), while it was not detected in Th1 cells (red line), non-polarized cells (solid black line) and isotype control (dotted green line)**.** This experiment was performed five times with DCs of B6 mice (n=1) and $CD4^+$ naïve cells from OT-II $Rag^{-/-}$ mice (n=3). Cells in each condition were cultured in triplicates.

3.2 Bcl-6, T-bet, Gata-3 and IFN-γ expression were detected in Tfh cells *ex vivo*

There are conflicting reports about the expression of Th1 and Th2 key regulatory transcription factors and the production of their corresponding cytokines, in Tfh cells. Therefore, and with the aim of characterizing the expression profile of Th1 and Th2 markers on putative "Tfh1" and "Tfh2", a bulk of Tfh cells were collected from both immunizing conditions and analyzed either by flow cytometry and RT-qPCR.

Subsequently, Tfh cells ($CD4^+CD44^+$ $CXCR5^+PD\text{-}1^+$), activated-Th cells ($CD4^+CD44^+$ $CXCR5^-PD\text{-}1^-$), non-activated Th cells ($CD4^+CD44^-CXCR5^-PD\text{-}1^-$), B cells ($CD19^+$ $FAS^-GL\text{-}7^-$) and GC B cells ($CD19^+$ $FAS^+GL\text{-}7^+$) were sorted from draining LNs of immunized mice. Expression of typical Th1, Th2 and Tfh transcription factors were evaluated on the described cells under OVA-CpG ODNs or OVA-IFA stimulation in both mouse backgrounds. Flow cytometry results shown are for Balb/c mice and are representative of the results obtained with the B6 mice.

As expected, CpG ODNs, a type 1 adjuvant, induced the expression of the Th1 major transcription factor, T-bet on activated-Th cells (blue dashed line) (Figure 4.8a). Expression of T-bet was also detected in Tfh cells (red solid line) in similar levels to those of activated-Th cells, and at higher levels when compared with non-activated Th cells (black solid line), as shown in figure 4.8a. Non-activated-Th cells were useful as a negative control for the expression of characteristic Th1 and Th2 markers. The typical Th2 major transcription factor Gata-3 was not detected in cells recovered from CpG ODNs stimulation (figure 4.8b). This data supports the idea that CpG ODNs induce a clean Th1 response, in line with the results from the ELISA assay (Figure 4.1).

IFA, a strong Th2 inducer, stimulated Gata-3 expression exclusively on activated-Th and Tfh cells (Figure 4.8e). Nonetheless, it is worthy of note that its expression could only be detected at low levels, as one would anticipate for *ex vivo* cultures. Importantly, even in the optimally stimulated *in vitro* cultures for Th2 polarization, the identification of Gata-3 by flow cytometry was somewhat modest as only some cells have acquired Gata-3 expression (Figure 4.7b).

Thus, *ex vivo* Gata-3 analysis should be performed on a homogeneous population, such as OVA-specific cells, which will allow the detection of Gata-3 in a more evident manner. T-bet expression could also be observed in Tfh cells under OVA-IFA stimulation, which confirmed that IFA, a strong Th2 inducer, also induced some features of Th1 response (Figure 4.8d). Subsequently, this adjuvant stimulated expression of T-bet and production of IgG2a by B cells, in low levels comparing with CpG ODNs stimulation, and when compared with the induced Th2 classical isotype antibodies (IgG1 and IgE).

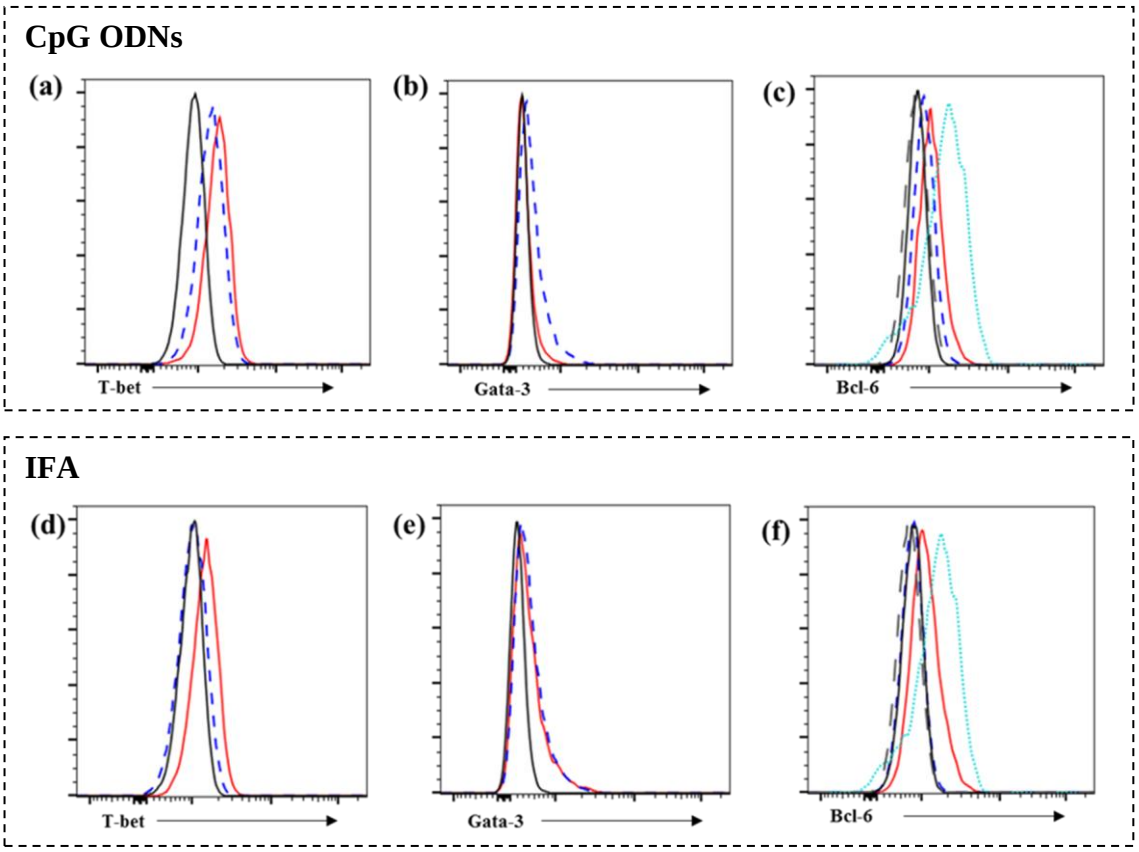


Figure 4.8- Expression of transcription factors in Balb/c mice immunized with OVA-CpG ODNs (a-c) and OVA-IFA (d-f). (a, d) T-bet expression and **(b, e)** Gata-3 expression on Tfh (solid red line), activated-Th (dashed blue line) and non-activated Th cells (solid black line) recovered from mice immunized with OVA-CpG and OVA-IFA, respectively; **(c, f)** Bcl-6 expression on Tfh (solid red line), activated-Th (dashed blue line), non-activated Th cells (solid black line), B cells (long dashed grey line) and GC B cells (dotted green line). This experiment was performed once in Balb/c mice (n=25) and B6 mice (n=12).

Moreover, the expression of the master transcription factor Bcl-6, a critical regulator of GC B cell and Tfh cell differentiation was also assessed (6,46). In this study, its expression was identified in Tfh and GC B cells recovered from mice immunized with OVA-CpG ODNs and OVA-IFA. Higher levels of Bcl-6 were detected in GC B cells (dotted green line), while Tfh cells (solid red line) expressed intermediate levels, comparing with B cells (dashed grey line) and non-activated Th cells (solid black line) (Figures 4.8c and 4.8f). This is in agreement with previous reports of elevated and intermediate Bcl-6 expression in GC B cells and Tfh cells of Bcl-6^{YFP} mice, respectively (72).

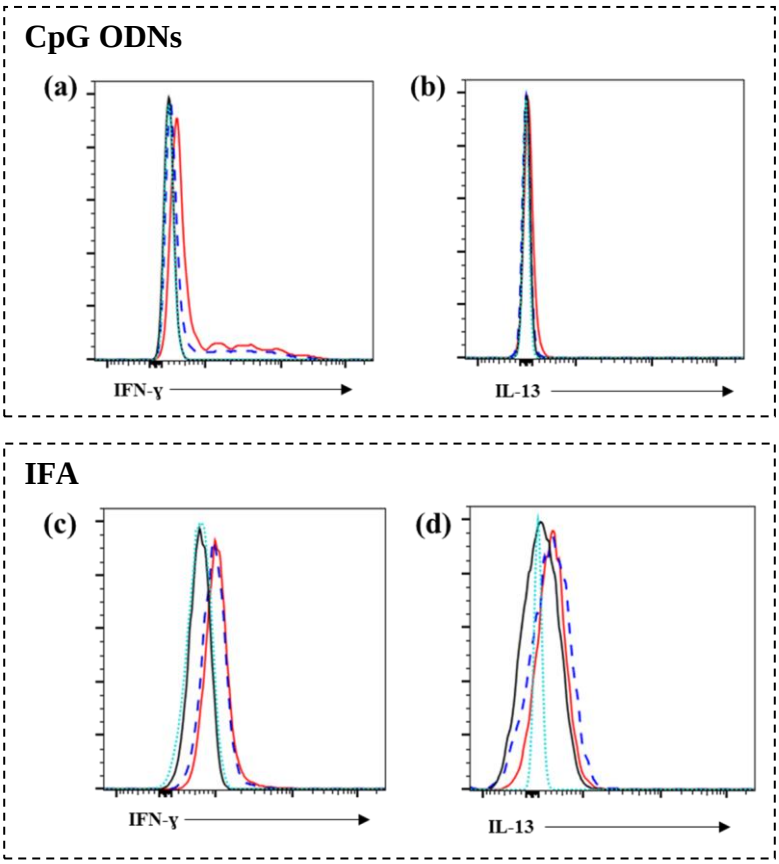


Figure 4.9- Production of cytokines in Balb/c mice immunized with OVA-CpG ODNs (a-b) and OVA-IFA (c-d). (a, c) IFN-γ production and **(b, d)** IL-13 production on Tfh (solid red line), activated-Th (dashed blue line) and non-activated Th cells (solid black line); Isotype control is shown as a dotted green line. This experiment was performed once in Balb/c mice (n=25) and B6 mice (n=12).

The cytokine profile of Tfh cells was equally analysed using flow cytometry. To guarantee the maximum cytokine production and accumulation, *ex vivo* T cells were cultured for 6 hours in stimulating conditions with PMA, Ionomycin, BFA and Golgi stop, in cRPMI at 37ºC and 5% CO_2.

Immunization with OVA-CpG ODNs stimulated the production of the characteristic Th1 cytokine IFN-γ by activated-Th cells (dashed blue line) as well as by Tfh cells (red solid line) (Figure 4.9a). The production of IFN-γ parallels the expression of T-bet in the same cell population. IL-13, a typical Th2 cytokine, was not detected in cells under type 1 adjuvant stimulation (Figure 4.9b)

Immunization with OVA-IFA also induced the IFN-γ production, although in low levels comparing with expression induced by OVA-CpG ODNs (Figure 4.9c). This data is in agreement with previous results showing that IFA, a type 2 adjuvant, induced the expression and production of typical Th1 markers. Importantly, OVA-IFA induced the production of low levels of IL-13 on activated-Th cells (dashed blue line) and Tfh cells (solid red line), when compared with the isotype control (dotted green line) (Figure 4.9d). As previously established in the Th2 polarization *in vitro* assay, the identification of IL-13 was more difficult, so the fact that the level of expression of IL-13 *ex vivo* was low, even following 4 hours of optimal *in vitro* stimulation, was indeed anticipated.

IL-4 was not possible to detect by flow cytometry, even in cells polarized *in vitro*. However, several studies have reported that IL-4 and IL-21 are produced by Tfh cells, collaborating to provide B cell help (43,55,73).

3.3 Bcl-6, Gata-3 and T-bet expression were detected in Tfh cells, *ex vivo*, through RT-qPCR

As described above, the RNA expression of key selected genes in elected target cells were compared using RT-qPCR. RNA from $5x10^4$ cells from each of the cell populations enumerated was extracted and cDNA synthesized using SuperScript II Reverse Transcriptase. CXCR5, Bcl-6, Gata-3, T-bet, IL-4 and IFN-γ target genes were amplified via qPCR using SYBR® Green.

The method of comparative relative quantification is based on the assessment of the expression level of a target gene in relation to a calibrator gene constitutively expressed, in this case β-2-microglobulin. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross a defined threshold. It is inversely proportional to the amount of target gene in the sample (i.e a lower Ct value indicates a greater amount of the target gene in the sample). A Ct value was obtained for each gene and the difference between the Ct of the target gene and the Ct of the calibrator gene is designated as the ΔCt. The fold difference is then calculated as 2 to the power of -ΔCt. In this study, $2^{-\Delta Ct}$ value allowed the comparative relative quantification of the expression of the same target gene in different cells populations. The $2^{-\Delta Ct}$ values are presented in a logarithmic scale for each cell population from mice immunized with OVA-CpG or OVA-IFA. Undetermined values were represented as non-detected (n.d) and mean that there was no amplification.

Typical Tfh (CXCR5 and Bcl-6), Th1 (T-bet and IFN-γ) and Th2 (Gata-3 and IL-4) markers were evaluated on Tfh cells recovered from bothimmunizing conditions, in order to establish similarities or differences in their expression, depending on how they had been primed..

According to the ΔCt method, the calculated relative expression level of CXCR5 in GC B cells, B cells and also in Tfh cells was between 1000-10000-fold higher than in Th cells, in both mouse backgrounds, immunized either with OVA-CpG ODNs or OVA-IFA (Figures 4.10a and 4.11a). As described in the literature, this surface chemokine receptor is highly expressed in Tfh cells, B cells and GC B cells, as confirmed in this study (49).

Bcl-6 was identified in high and intermediate levels on GC B cells and Tfh cells, respectively, from Balb/c mice, under both immunizing conditions (Figure 4.10b), in concordance with the flow cytometry results. Expression of Bcl-6 was equally identified in GC B cells collected from B6 mice under both immunizing conditions, although at lower levels comparing with Balb/c mice. However, only Tfh cells stimulated with OVA-IFA expressed Bcl-6 (Figure 4.11b).

Amplification of the Th1 key transcription factor T-bet was noted in all cell populations recovered from Balb/c mice stimulated with both immunizing conditions (Figure 4.10c). CpG ODNs, induced a significant expression of T-bet in activated-Th cells and Tfh cells, as previously observed by flow cytometry. Additionally, it was also demonstrated that IFA, a strong Th2 adjuvant, also induces some Th1 response with a consequent production of T-bet on activated-Th cells (Figure 4.10c). However, T-bet was highly expressed in B cells, GC B cells and non-activated Th cells ($CD44^-$) stimulated with OVA-IFA, comparing with OVA-CpG ODNs, which was not expected, even more in non-activated Th cells.

Expression of T-bet was detected mostly and at higher levels in cells from B6 mice when immunized with OVA-CpG ODNs (Figure 4.11c). More specifically, there was a 4000-fold and 100-fold increase of T-bet amplification in activated-Th cells and Tfh cells in relation to non-activated Th cells, respectively. T-bet was also detected in B cells and GC B cells (Figure 4.11c) and accordingly with the literature, as expression of T-bet in B cells is related with IgG2c isotype switching, characteristic of Th1 responses (74). The huge increase of T-bet expression in Tfh cells from B6 mice immunized with OVA-CpG ODNs, may have had an impact in decreasing expression of Bcl-6 in the same cells, not allowing its detection by RT-qPCR. Analysis through RNA-sequencing will provide more information related with the co-expression and interaction between these distinct transcription factors.

Finally, Gata-3 was detected at low levels in activated-Th cells and also in B cells from Balb/c mice immunized with type 1 or type 2 adjuvants (Figure 4.10d). This result was not expected and should be confirmed by a different approach.

Balb/c mice

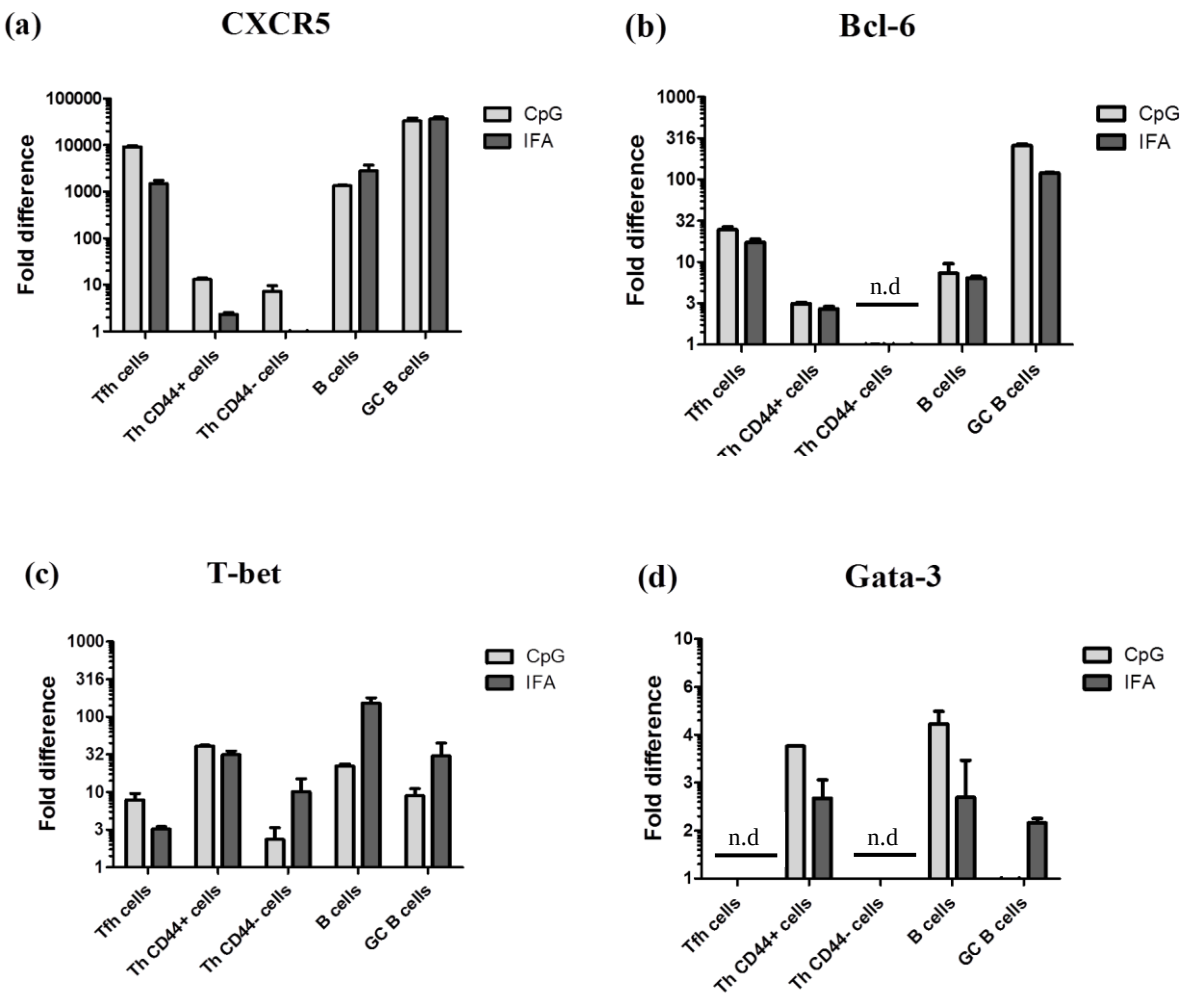


Figure 4.10- Comparative relative quantification of CXCR5, Bcl-6, Gata-3 and T-bet genes in Tfh, activated-Th (CD44^{+}), non-activated-Th (CD44^{-}), B cells and GC B cells of Balb/c mice. Target genes **(a)** CXCR5, **(b)** Bcl-6, **(c)** T-bet and **(d)** Gata-3 were amplified using the RT-qPCR method and the Ct value was obtained for each calibrator and target genes. Gene expression in each cell population was assessed using the following formula: $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{calibrator gene}}$; Fold difference= $2^{-\Delta Ct}$. This experiment was performed once in Balb/c mice (n=25).

B6 mice

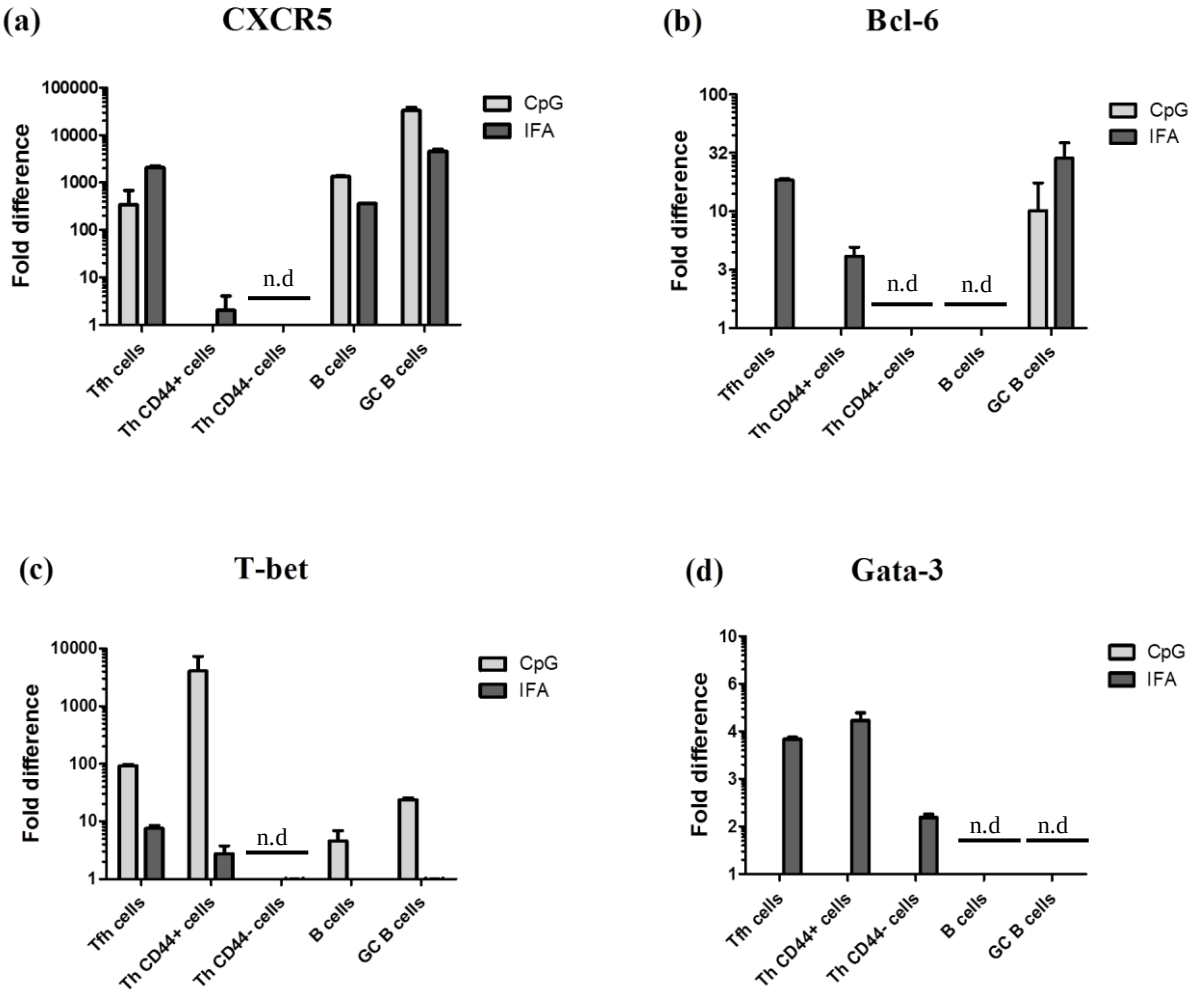


Figure 4.11- Comparative relative quantification of CXCR5, Bcl-6, Gata-3 and T-bet genes in Tfh, activated-Th (CD44^{+}), non-activated-Th (CD44^{-}), B cells and GC B cells of B6 mice. Target genes **(a)** CXCR5, **(b)** Bcl-6, **(c)** T-bet and **(d)** Gata-3 were amplified using the RT-qPCR method and the Ct value was obtained for each calibrator and target genes. Gene expression in each cell population was assessed using the following formula: $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{calibrator gene}}$; Fold difference= $2^{-\Delta Ct}$. This experiment was performed once in B6 mice (n=12).

For instance, Gata-3 transcripts in OVA-specific cells could be amplified using absolute quantification for RT-qPCR. This way, the target gene of interest can be directly quantified with precision by the number of digital PCR replicates or using a standard curve method, where comparative analysis between a target and a reference gene is needless (75).

Notably, amplification of Gata-3 in B6 mice was detected only in activated-Th and Tfh cells with OVA-IFA immunization (figure 4.11d) and at similar levels to the ones referred in a study analysing Tfh cells and Th2 cells in response to helminth antigens (76). Comparable to our study, the presence of a PD-1^+ subset of Th2 cells in mice during chronic schistosomiasis, absent in naïve mice, has been previously reported (76). In order to evaluate whether IL-4/GFP^+CD4^+Th2 cells that also expressed PD-1 are a Tfh cell population, authors made use of 4get/ KN2 mice in which IL-4–competent Th2 cells are marked by GFP and IL-4–producing cells additionally express huCD2 on their surface (76).

These animals were immunized with Soluble Egg Antigen (SEA), and at day 14, draining LNs cells were stained for PD-1, CXCR5 and ICOS. To further define the PD-1^+ IL-4/ GFP^+ $CD4^+$ T cells, they purified them by FACS and used RT-qPCR to assess expression of Bcl-6, IL-21, IL-4 and Gata-3. The same techniques were used in an attempt to characterize the phenotype of Tfh cells under different immunizing conditions.

Transcript levels of Gata-3, were comparable in Th2 (PD-1^-GFP^+ $CD4^+$) and Tfh cells (PD-1^+GFP^+ $CD4^+$) from SEA-immunized mice. Moreover, both Tfh and Th2 cells expressed IL-5 and IL-13 (76). Similarly, Tfh and activated-Th cells, recovered from B6 mice immunized with OVA-IFA, expressed comparable levels of Gata-3.

Moreover, supporting our data, a study focusing on the development and fate of Tfh cells on *Il21*$^{GFP/+}$ mice, similarly reported Tfh cells as multifunctional helper cells that co-expressed transcription factors and several cytokines related to the Th1 and Th2 subsets. *Il21*$^{GFP/+}$ reporter mice were immunized with NP-KLH in alum and the authors assessed the expression of transcripts encoding T-bet and Gata-3. While T-bet was more abundant in IL-21–GFP^+ Tfh cells than in their IL-21–GFP^- counterparts, transcripts encoding Gata-3 were low in both populations (77).

Equally, high levels of T-bet were detected in Tfh cells in Th1-biased response, comparing with low levels of Gata-3 on Th2 immunizing conditions. Additionally, Tfh cells expressed transcripts encoding IL-4 in the Th2-biased *Il21*$^{GFP/+}$ mice immunization with NP-KLH. Also, IFN-γ was the main cytokine secreted by Tfh cells during infection with influenza virus in *Il21*$^{GFP/+}$ reporter mice (77).

Significantly, we reported that the transcriptional factor and cytokine profile of Tfh cells were similar to activated-Th cells, depending on the priming conditions. Tfh cells from B6 mice co-expressed Bcl-6 and T-bet when stimulated with OVA-CpG ODNs, which induced IFN-γ production in similar levels to the ones produced by activated-Th cells. OVA-IFA immunization induced expression of similar Gata-3 levels in Tfh and activated-Th cells. This data could preliminarily indicate that Tfh cells can be divided in subsets, putative "Tfh1" and "Tfh2" cells, which share several genes with their corresponding functional effector Th1 and Th2 cells, respectively.

Expression of Th1 and Th2 cytokines, namely IFN-γ and IL-4, was not detectable, even in activated-Th cells (data not supplied). Thus, it would have been important to stimulate the cells with PMA and Ionomycin in order to induce *ex vivo* and support cytokine production, and also with BFA and Golgi stop to block secretory pathways, accumulating cytokines inside the cells. Consequently, RNA levels of IFN-γ and IL-4 might have increased, allowing their detection via RT-qPCR. In the literature, there are reports assessing the production of IL-4, IFN-γ and IL-10 on Tfh cells, using RT-qPCR (77).

5. Discussion and Conclusions

Tfh cells were initially described as a subset of $CD4^+$ T cells expressing high levels of CXCR5 and Bcl-6 as key transcription factor (31). Inside the GC, Tfh cells provide help and support to B cells during affinity maturation and class switch recombination pivotal for antibody-producing plasma cells and memory B cells differentiation (44). Tfh cells express a diverse repertoire of other surface molecules, namely PD-1, CD40L and ICOS, and also produce several cytokines including IL-21 and IL-4 which are critical in driving B-cell proliferation, survival, and isotype class switch (51). Additionally, Tfh cells are also referred in the literature as $CD25^-$ and it is now known that they express high levels of the activation marker CD44.

The role of Tfh cells (or of putative "Tfh1" and "Tfh2" subsets) on driving GC reaction and selection of Ig isotypes, as well as the influence of Th1 and Th2 cells in this process, depending on the diversity of the antigens, remains to be clarified. This way, adjuvants can effectively improve and modulate humoral and cellular immune responses against the antigen. CpG ODNs, a type 1 adjuvant, prompted cells into a Th1-biased response that induced isotype switching to IgG2a. This adjuvant was selected in detriment of TMX, SAS or Montanide ISA 720 VG, as it induced a clean Th1 response in both Balb/c and B6 mice. IFA is a type 2 adjuvant that allowed the slow release of OVA, inducing an effective Th2 polarization characterized by IgG1 and IgE production.

Cell recruitment in response to OVA antigen was also dissimilar depending on adjuvant type. CpG ODNs was a weak stimulant of the GC reaction since the majority of OVA-specific cells remained outside of GC. Thus, less Tfh cells were recovered, which motivated the increase in the number of transferred OVA-specific cells. Immunization with OVA-IFA efficiently recruited OVA-specific $CD4^+$ T cells into the GC in both Balb/c and B6 mice, where Tfh cells can provide help to B cells for an effective production of IgG1 and IgE. Adoptive cell transfers constitute a pivotal approach in this study, as it enabled recovery of OVA-specific cells in order to pursue the additional molecular characterization of putative "Tfh1" and "Tfh2" populations through RNA-sequencing analysis.

In parallel, in an effort to identify expression of Th1 and Th2 markers on Tfh cells, an *ex vivo* analysis was performed via flow cytometry and RT-qPCR.

In our study, the Th1 transcription factor T-bet was detected in total Tfh cells ($CD4^+CD44^+CXCR5^+PD\text{-}1^+$), that also expressed Bcl-6. Notably, this phenotype developed mostly when mice were immunized with OVA-CpG ODNs, a type 1 adjuvant. Tfh cells also produced IFN-γ in similar levels comparing with activated-Th cells. The identification of Th2 markers in Tfh cells was challenging, either using flow cytometry or the RT-qPCR method. Low levels of Gata-3 transcripts were detected in Tfh cells of OVA-IFA immunized B6 mice.

Importantly, it has been known that different mouse strains can display distinct susceptibility to Th1 and Th2 polarization, and significant differences were observed in the way that Balb/c (more prone to Th2) and B6 mice (more prone to Th1) responded to type 1 or type 2 adjuvants. Cells recovered from B6 mice expressed T-bet and produced IFN-γ, exclusively when stimulated by OVA-CpG ODNs, whereas expression of Gata-3 in Tfh cells was detected when cells were polarized with OVA-IFA. This data, in agreement with the ELISA assay, confirmed that the B6 mouse background produced more skewed responses.

In Balb/c mice, and in obvious contrast, both immunizing conditions induced the expression of Gata-3 and T-bet, even though at low levels. In fact, IFA, a strong type 2 adjuvant, also induced some Th1 response, characterized by T-bet expression, IFN-γ production and IgG2a class switch. To maybe overcome this, the same approach should be performed in OVA-specifc Tfh cells, a much more homogenous population, in order to verify whether the same phenomenon would occur in cells recoverd from Balb/c mice. This analysis would allow *ex vivo* validation of the currently ongoing RNA-sequencing study.

Our access to equivalent populations from two distinct genetic backgrounds will facilitate the bioinformatics analysis as the critical transcripts should be shared between putative Tfh1 populations from B6 and Balb/c mice, while absent from Tfh2 populations from the two backgrounds, and vice-versa. This allows molecular confirmation that any given phenotypic molecular signature is attributable to effector function, *e.g*. Tfh1 vs Tfh2, and not to some particular mouse background feature.

Together, these results have already provided good indications to discover the existence of Tfh cell subpopulations, presumed putative "Tfh1" and "Tfh2". Nevertheless, this study does not suffice by itself to prove that Tfh cells can be subdivided into different subsets depending on the immunizing conditions. To further substantiate these results, valuable information will be delivered from the RNA-sequencing analysis being performed (Genecore, EMBL) and subsequent bioinformatics analysis. It will allow the discrimination of the gene expression profiles between OVA-specific putative "Tfh1" and "Tfh2" and also of OVA-specific activated-Th1 and -Th2 cells.

Recent studies have reported the existence of Tfh subpopulations, both in mice and humans. Joe Craft and colleagues (78) defined three different Tfh populations ($CD4^{+}CD44^{hi}CXCR5^{hi}PD\text{-}1^{hi}$) based on IL-21 and IL-4 production. They infected $Il21^{Kat/Kat}$ $Il4^{GFP/GFP}$ mice (B6 background) with the helminth *N. brasiliensis*, which induces IL-4 production in Tfh cells. This is a multi-antigenic infection that will induce a strong Th2 response, while we are inducing a clean Th2 response against OVA protein polarized by IFA type 2 adjuvant. Researchers, collected the cells at day 8, whereas we decided to collect at day 11, the peak of GC reaction.

They found that the majority of Tfh cells secreted only one (Tfh21) or the other cytokine (Tfh4), and relatively few Tfh cells secreted both IL-21 and IL-4 (Tfh21+4). Additionally, the authors determined that Tfh21, Tfh21+4 and Tfh4 populations are transcriptionally distinct from each other using RNA-sequencing, the same technique that we are using to characterize putative "Tfh1" and "Tfh2" cells. Craft et al, identified 1,300 genes differentially expressed, and reported that the Tfh21 and Tfh21+4 cells were most alike, whereas Tfh4 cells shared similarities with the Tfh21+4 and the Th2 populations (78). In accordance, we preliminarily identified the expression of T-bet, IFN-γ and Gata-3 in Tfh cells ($CD4^{+}CXCR5^{+}PD\text{-}1^{+}Bcl\text{-}6^{+}$) in similar levels to corresponding Th1 or Th2 cells. This data could indicate that Tfh and Th cells could share some similarities in the same priming condition.

Moreover, Hideki Ueno identified different circulating Tfh cell (cTfh) subsets on human blood (79). $CXCR5^{+}CD4^{+}$ cTfh do not express Bcl-6 transcription factor, and how cTfh cells maintain Tfh-like properties is currently uncharacterized.

The combination of CXCR3 and CCR6, chemokine receptors preferentially expressed by Th1 cells and Th17, respectively, defines three major subsets within cTfh cells: cTfh1 cells ($CXCR3^{+}CCR6^{-}$ cells), cTfh2 cells ($CXCR3^{-}CCR6^{-}$ cells) and cTfh17 cells ($CXCR3^{-}CCR6^{+}$ cells). cTfh1 cells express the transcription factor T-bet, and produce IFN-γ, while cTfh2 cells express the transcription factor Gata-3, and produce IL-4, IL-5, and IL13. cTfh17 cells express the transcription factor RORγt, and produce IL-17A, IL-17F and IL-22. In patients with juvenile dermatomyositis, adult systemic lupus erythematosus, rheumatoid arthritis, and Sjogren's syndrome, cTfh1 cells are underrepresented among cTfh cells, whereas cTfh2 and/or cTfh17 cells are overrepresented (79).

Concluding remarks and future perspectives

This study showed that adjuvants can be differentially administrated in order to modulate and stimulate immune responses against OVA protein. CpG ODNs, TLRs-dependent adjuvant, stimulated T-bet and IFN-γ expression on T cells and induced isotype switching to IgG2a. On the other hand, OVA-IFA efficiently induced an effector Th2 response characterized by high levels of OVA-specific IgG1 and IgE. Additionally, it was shown that adoptive cell transfers of OVA-specific $CD4^{+}$ T cells from OT-II $Rag^{-/-}$ and DO11.10 $Rag^{-/-}$ mice into congenic mice serve as an appropriate model for recovery of a homogenous population of OVA-specific Tfh and Th cells. Interestingly, co-expression of Bcl-6 and other Th1 and Th2 markers on Tfh cells was demonstrated, which contributed with data pointing to the existence of presumed "Tfh1" and "Tfh2" cells.

Briefly, data from the RNA-sequencing analysis of putative "Tfh1" and "Tfh2" subsets will be available and may, therefore, unravel how Th1, Th2 and Tfh cells drive GC reactions producing specialized class of Ig and the key molecular mechanisms involved. In addition, the identification of those mechanisms will potentially enable the development of therapeutic strategies specifically targeting Tfh contribution in Th1 or Th2 diseases, e.g. allergy.

In order to confirm the significance of results obtained following RNA-sequencing analysis, phenotypic studies with mouse models of Th1 and Th2 immune responses, as

well as with clinical samples will be performed. Based on the transcriptome analysis and phenotypic studies, candidate genes that are likely to be important for Tfh function associated with Th1 and Th2 responses can be investigated. The functional relevance of those candidate molecules will be validated *in vitro* and *in vivo*, leading to an understanding of mechanisms underlying type 1 and type 2 Tfh help.

It would be interesting to collect OVA-specific cells from mice immunized with Th1, Th2 and also with non-polarizing conditions (type 1 and type 2 adjuvants together) and co-culture with B cells. After stimulating the cells, the readout would be cytokine secretion, which could be measured by ELISA, flow cytometry and RT-qPCR, and production of different classes of Ig, determined by ELISA.

Even more, *in vitro* co-cultures of defined populations of B and Tfh cells in the presence of inhibitors, blocking antibodies, or gene knock-down would reveal interesting to assess the functional impact of a specific gene product/pathway.

Further characterization of mouse Tfh1 and Tfh2 cells, *ex vivo*, and evaluation of the expression of surface markers, namely CXCR3, CCR4 and CCR6, might prove beneficial in the definition of distinct effector Tfh populations, as it is done in humans.

In addition, for *in vivo* validation, the proposal would be to use a mouse model of OVA-immunization under type-1 or type-2 conditions and Bcl-6-Cre mice to ablate candidate genes on Tfh cells. Moreover OVA-specific Tfh1 and Tfh2 cells could be sorted from mice immunized with type 1 or type 2 adjuvants, respectively, and separately transferred to Bcl-$6^{-/-}$ or CXCR$5^{-/-}$ mice (on T cells). Recipient mice will be immunized with the type 1 or type 2 adjuvant in order to evaluate the plasticity and memory of the defined Tfh cell subsets.

Apart from Tfh cell subpopulations, addressing the study of expression of CD44 in Tfh cell throughout the GC development, as well as its impact on Tfh differentiation and function would also be interesting and, most likely, relevant.

In addition to the mouse studies it will be important to characterize Tfh populations isolated from tonsils of atopic and non-atopic individuals. With human tonsils it would be possible to investigate the expression of key genes, identified through bioinformatics methods, using flow cytometry or RT- qPCR among sorted Tfh cells.

6. References

6. References

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7. Attachment

1. Adoptive cell transfer from OT-II Rag$^{-/-}$ mice into B6 Thy1.1 recipient mice was not successful

Having established optimal Th1 and Th2 immunizing conditions, the objective was to isolate antigen specific Tfh and activated Th cells. For this, cells from OT-II Rag$^{-/-}$ mice were transferred into B6 Thy1.1 recipient mice. The following day, mice were immunized in the hind footpads with OVA protein in conjugation with the defined immunizing adjuvants to polarize for Th1 and Th2 responses. However, the adoptive cell transfer from OT-II Rag$^{-/-}$ mice into B6 Thy1.1 mice was not successful, since at day 11 during cell sort, OVA-specific Tfh and Th cells (Thy1.2^{+}Vβ5^{+}) were not detected, either in mice immunized with CpG ODNs or IFA adjuvant (Figures 7.1a and 7.1b).

Nevertheless, antibody titres measured by ELISA (Figure 7.2) indicated that an immune response occurred in these mice. High titres of IgG2a as well as of IgG1, were detected in the serum of mice immunized with OVA-CpG ODNs. Also, unexpectedly and to our surprise, IgG2a presented levels superior in mice immunized with OVA-IFA to those in immunized with CpG ODNs. Immunization with IFA induced the production of high titres of IgG1 and IgG2a (Figure 7.2).

Several possibilities were tested in an effort to understand and tackle this situation. The phenotype of OT-II Rag$^{-/-}$ cells was confirmed yet again and it was observed that all CD4^{+} T cells were Thy1.2^{+}Thy1.1^{-}Vβ5^{+}Vα2^{+} and that CD19^{+} B cells were not detected, as expected (data not supplied). Second, the mixture of antibodies were re-tested by flow cytometry and they were all functional. Thirdly, to exclude a contamination in the adjuvants used, new vials were opened and used to immunize naïve B6 Thy1.1 mice (without cell transfer) and cells from draining LNs stained with the antibody mix previously used. In this particular test experiment, high titres of IgG2a and IgG1 were measured in OVA-CpG ODNs or OVA-IFA immunization, respectively, and as expected. Higher percentages of Tfh (CD4^{+}CXCR5^{+}PD-1^{+}) and GC B (CD19^{+}GL-7^{+}FAS^{+}) cells were also detected, via flow cytometry (data not supplied).

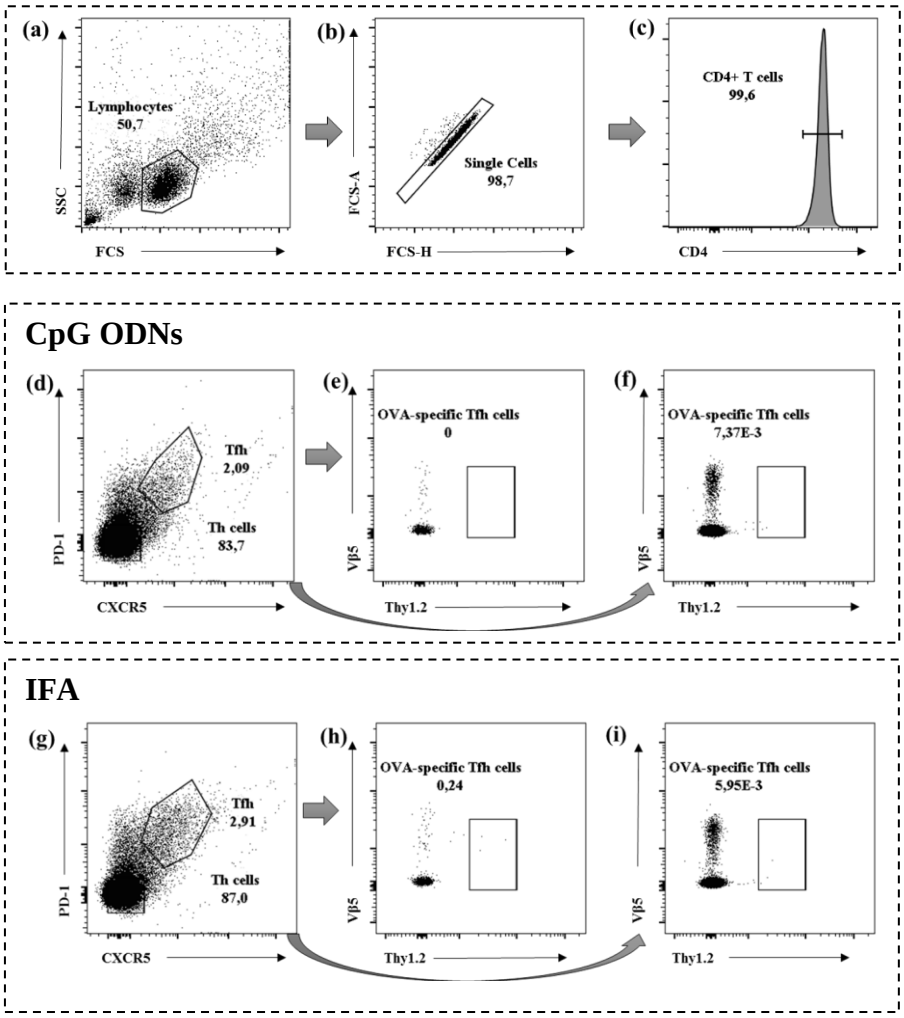

Figure 7.1- Gating strategy for OVA-specific Tfh cells identification, from draining LNs of B6Thy1.1 mice under both immunizing conditions. (a) Lymphocytes population **(b)** Doublet cells exclusion **(c)** $CD4^+$ T population; (a-c) gating strategy is the same for both adjuvants; **(d, g)** Tfh cells ($CXCR5^+PD\text{-}1^+$) and Th cells outside the GC ($CXCR5^-PD\text{-}1^-$); **(e, h)** OVA-specific Tfh cells ($Thy1.2^+V_\beta5^+$) was not detected as well as **(f, i)** OVA-specific Th cells ($Thy1.2^+V_\beta5^+$). **(d-f)** results from mice immunized with OVA-CpG ODNs (n=8); **(g-i)** results from mice immunized with OVA-IFA (n=6). This experiment was performed four times.

Moreover, in a parallel experiment, Balb/c mice received DO11.10 $Rag^{-/-}$ cells and were immunized with the same recently opened adjuvants vials. As shown in figure 4.2 and table 4.1, OVA-specific cells were detected and could be recovered from Balb/c animals, reinforcing, yet again, that there was no problem either with the adjuvants or the antibodies used. Thus, these hypothesis were totally excluded.

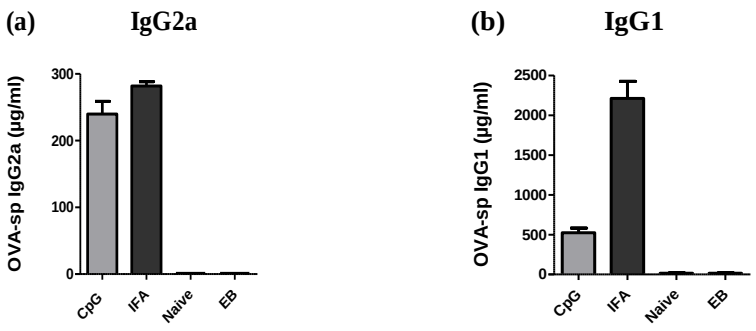

Figure 7.2- Titres of IgG2a, IgG1 measured on day 14 in serum of B6 Thy1.1 mice immunized with OVA and the indicated adjuvants. **(a)** OVA-specific IgG2a, **(b)** OVA-specific IgG1 analysed in mice serum (indicated as concentration at µg/ml for IgG). Ig measured in B6Thy1.1 mice immunized with CpG ODNs (n=8) and IFA (n=6) and non-immunized mice, designed as naïve (n=2); This experiment was performed four times and each sample tested in duplicates.

The following approach was to perform skin transplants from OT-II $Rag^{-/-}$ onto B6 Thy1.1 mice to evaluate the rejection that one would anticipate considering the inability to detect the OT-II $Rag^{-/-}$ transferred cells, in the adoptive cell transfer approach.

Full-thickness skin was collected from tails of OT-II $Rag^{-/-}$ mice (Group 1), OT-II $Rag^{+/+}$ mice (Group 2) and B6 Thy1.1 (Control Group) donor mice and placed in sterile saline solution until the time of transplant. Then, B6 Thy1.1 recipient mice were anaesthetized with subcutaneous (s.c) injection of ketamine (75mg/KgBW, Imalgene 100, Merial) and medetomidine (1mg/KgBW, Domtor, Pfizer) in saline solution.

B6 Thy1.1 recipient mice were placed with the left flank exposed, swabbed with 70% (v/v) ethanol and shaved with an electric shaver to expose the skin. A square shaped incision (approximately 1cm x 1cm) was made with a scissors and filled with a size-matched graft of the collected donor skin tail. After sealing with Vetbond™ Tissue Adhesive (Santa Cruz Biotechnology), the graft was covered with Betadine® (Iodopovidone ointment, 100mg/g, MedaPharma), Grassolind (Hartmann) and wrapped in autoclave tape. Anaesthesia was reverted with s.c. injection of atipamezole (1mg/KgBW, Antisedan, Pfizer).

The bandage was removed on day 8 post-transplant and the skin grafts were inspected daily. Graft rejection was defined when tissue became necrotic, and if the transplant survived for 30 days without evidence of necrosis, it was considered accepted.

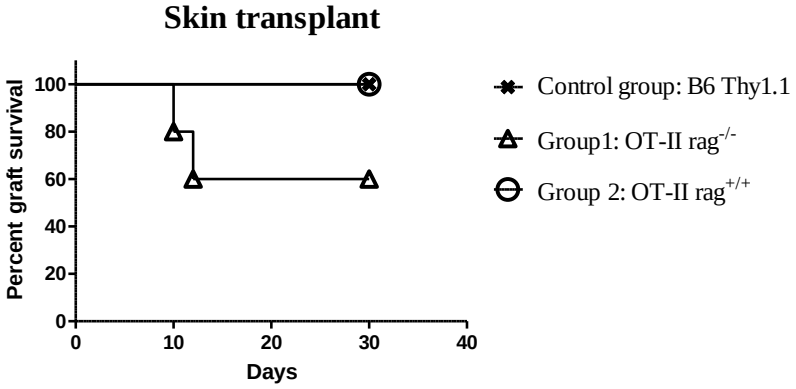

Figure 7.3- Skin transplant of OT-II Rag$^{-/-}$ was rejected by B6 Thy 1.1 mice.
The control group received B6 Thy1.1 skin grafts (n=2); OT-II Rag$^{-/-}$ skin (Group 1) were transplanted onto B6 Thy1.1 mice (n=5) and OT-II Rag$^{+/+}$ skin (Group 2) were transplanted onto B6 Thy1.1 mice (n=4). This experiment was performed once.

The control group accepted the skin grafts, because the tissue remained without evidence of necrosis for more than 30 days, as expected. In group 1, two acute rejections were observed, on days 10 and 12, as the tissue became necrotic, accounting for 40% graft rejection. Worthy of note, the other 3 mice in the group displayed some difficulty in wound healing around the edges of the graft, with some signs of inflammation present; nevertheless, the graft was not eliminated and by day 30 the graft survived without evidence of necrosis. Group 2, as expected, accepted the skin grafts, however some signs of inflammation were observed as well as some difficulty in wound healing around the edges of the graft, as group 1.

Therefore, to overcome this surprising obstacle, a new breeding was initiated crossing B6 Thy1.1 with B6 Rag$^{-/-}$ mice (Thy1.2), to establish an immunocompetent mice that can receive cells from OT-II Rag$^{-/-}$ mice, without rejection.

Then, cells from OT-II Rag$^{-/-}$ mice were adoptively transferred into the new recipient B6 Thy1.1/Thy1.2 mice. As can be visualized in figure 4.3, OVA-specific Tfh cells ($CD4^+CD44^+$ $CXCR5^+PD\text{-}1^+$ $Thy1.2^+$ $V\beta5^+V\alpha2^+$) and OVA-specific activated-Th cells ($CD4^+CD44^+$ $CXCR5^-PD\text{-}1^-$ $Thy1.2^+$ $V\beta5^+V\alpha2^+$) could be identified and collected 11 days after immunization in the hind footpads with OVA-CpG ODNs or OVA-IFA, respectively. At this stage, all the conditions were set to recovery of OVA-specific Tfh and OVA-specific activated-Th cells from B6 mice for RNA-seq analysis.

2. Tables

Table 7.1- Monoclonal antibodies (mAbs) used in cell surface stainings.

mAb (mouse)	Conjugate	Vendor	Catalogue number (Cat. No)	Work dilution
Rat Anti-CXCR5	Biotin	BD Bioscience	551960	1:50
Mouse Anti-DO11.10 TCR	Biotin	eBioscience	13-5808-82	1:100
Mouse Anti-DO11.10 TCR	FITC	eBioscience	11-5808-82	1:100
HamsterAnti-TCR-β	FITC	eBioscience	11-5961-82	1:100
Rat Anti- CD86 (B7-2)	FITC	eBioscience	11-0862-82	1:100
Mouse Anti-V_β5.1 5.2	FITC	BD Bioscience	553189	1:100
Rat Anti-CD4	PE	eBioscience	12-0041-85	1:400
Hamster Anti-CD95 (Fas)	PE	BD Pharmingen	554258	1:100
Hamster Anti-CD11c	PE	eBioscience	17-0114-82	1:400
Hamster Anti-CD279 (PD-1)	PE	eBioscience	12-9985-82	1:100
Rat Anti-CD4	PerCP-Cy5.5	eBioscience	45-0042-82	1:400
Streptavidin	PerCP-Cy5.5	eBioscience	45-4317-82	1:100
Hamster Anti-CD279 (PD-1)	PE-Cy7	eBioscience	25-9985-82	1:100
Rat Anti-CD90.2 (Thy1.2)	PE-Cy7	eBioscience	25-0902-81	1:1600
Rat Anti-CD4	eFluor® 660	eBioscience	50-0041-82	1:100
Rat Anti-CD44	APC	BioLegend	103012	1:400
Rat Anti-CD25	APC-eFluor® 780	eBioscience	47-0251-82	1:100
Rat Anti-CD4	APC-eFluor® 780	eBioscience	47-0042-82	1:100
Rat Anti-CD19	eVolve™ 605	eBioscience	83-0193-42	1:100
Rat Anti- TCR V_α2	eFluor® 450	eBioscience	48-5812-82	1:100

Rat Anti-CD4	Pacific Blue™	BioLegend	116008	1:100
Rat Anti-CD4	Brilliant Violet 510™	BioLegend	100559	1:800
Live/ Dead® Fixable Near	Amcyan	Alfagene/ Life Technologies	L10119	1:500
Rat Anti-CD44	Brilliant Violet 605™	BioLegend	103047	1:100
Rat Anti-CD90.2 (Thy1.2)	Brilliant Violet 605™	BioLegend	140317	1:200
Rat Anti- CD3	Brilliant Violet 711™	BioLegend	100241	1:50
Streptavidin	Brilliant Violet 711™	BioLegend	405241	1:100

Table 7.2- Monoclonal antibodies (mAbs) used in intracellular stainings.

mAb (mouse)	Conjugate	Vendor	Cat. No	Work dilution
Mouse Anti-Bcl-6	Alexa 488	BD Biosciences	561524	1:20
Rat Anti-IFN-γ	FITC	eBioscience	11-7311-82	1:100
Rat Anti-IL-13	PE	eBioscience	12-7133-80	1:50
Rat Anti- Gata-3	PE	eBioscience	12-9966-42	1:20
Mouse Anti-T-bet	PerCP-Cy5.5	eBioscience	45-5825-82	1:100
Rat Anti-IL-21	APC	eBioscience	17-7211-80	1:20
RatAnti-IL-4	APC	eBioscience	17-7041-82	1:20

Table 7.3- Monoclonal antibodies (mAbs) used as isotype control.

mAb (mouse)	Conjugate	Vendor	Cat. No	Work dilution
Rat Anti-IgG1	FITC	eBioscience	11-4301	1:100
Mouse Anti-IgG1	PE	eBioscience	12-4714-82	1:100
Rat Anti-IgG1	APC	eBioscience	17-4301-81	1:100